HABILITATIONSSCHRIFT

Genetische Epidemiologie krankheitsrelevanter Messwerte in der Allgemeinbevölkerung: QTL-Analysen an Zwillingen

Zur Erlangung der Lehrbefähigung für das Fach
Genetische Epidemiologie

vorgelegt der Medizinischen Fakultät Charité
der Humboldt-Universität zu Berlin

von
Dr. rer.nat. Andreas Busjahn
aus Berlin

Dekan: Prof. Dr. Joachim W. Dudenhausen

Gutachter: 1. Herr Prof. Dr. Baur/Bonn

2. Herr Prof. Dr. Vogel/Leimen

Datum der Habilitation: 26.03.2002

Zusammenfassung

Das Jahr 2000 wird oft als Meilenstein der Entwicklung der Humangenetik bezeichnet. Eine Relevanz für die praktische Medizin erlangt das Humangenom-Projekt jedoch erst, wenn die Funktion der einzelnen Gene in komplexen physiologischen Systemen und die genetische Variabilität aufgeklärt sind.

Die hier vorgelegten Studien beruhen auf der Annahme, dass der Einfluss genetischer Variabilität nicht nur im Vergleich kranker und gesunder Menschen sichtbar wird, sondern auch in der Variabilität physiologischer Parameter in der Allgemeinbevölkerung nachweisbar ist. Grundlage aller Studien war eine medizinische Untersuchung von gesunden eineiigen und zweieiigen Zwillingspaaren. Es wurde für Kennwerte des Herz-Kreislauf-Systems die Stärke genetischer Einflüsse (Heritabilität) bestimmt. Weiterhin erfolgten Kopplungs- und Assoziationsanalysen mit ausgewählten Kandidatengenen.

Der Einfluss spezifischer Gene auf die Blutdruckregulation, die Herzgröße, EKG-Parameter sowie Blutfette konnte nachgewiesen werden. Weiterhin wurde der prinzipielle Nachweis erbracht, dass die funktionelle Untersuchung einzelner Gene in unausgelesenen Stichproben realisierbar ist.

Abstract

The year 2000 is often called a milestone in the history of human genetics. The knowledge of the sequence of the human genome will only become relevant for clinical medicine when the function of genes within complex physiological systems as well as the genetic variability will be revealed.

The studies reported here are based on the assumption that the influence of genetic variability does not only become obvious by comparison of affected and unaffected subjects but is as well detectable in the variability of physiological parameters in the general population. All studies are based on testing healthy mono- and dizygotic twins. We determined the heritability of various cardiovascular parameters. Furthermore selected candidate genes were tested by linkage and association analyses.

We could demonstrate the influence of specific genes on blood pressure regulation, heart size, ECG and lipids. These studies are a proof of principle for the functional analysis of single genes in unselected random samples.

Schlagwörter:

Genetische Epidemiologie, Zwillinge, Kopplung, Assoziation, Blutdruck, Lipide, EKG

Keywords:

genetic epidemiology, twins, linkage, association, blood pressure, lipids, ECG

Diese Habilitationsschrift beruht auf dem Inhalt der nachfolgenden Veröffentlichungen, die im Anhang zusammengestellt sind:

Busjahn A, Faulhaber HD, Viken RJ, Rose RJ, Luft FC. Genetic influence on blood pressure with the cold-pressor test: A twin study. J Hypertens, 1996, 14, 1195-1199.

Becker A, **Busjahn A**, Faulhaber HD, Bähring S, Robertson J, Schuster H, Luft FC. Twin zygosity, Automated determination with microsatellites. J Reprod Med, 1997, 42, 260-266.

Busjahn A, Knoblauch H, Knoblauch M, Bohlender J, Menz M, Faulhaber HD, Becker A, Schuster H, Luft FC. Angiotensin-converting enzyme and angiotensinogen gene polymorphisms, plasma levels, cardiac dimensions: A twin study. Hypertension,29 [part 2], 1997, 165-170.

Knoblauch H, **Busjahn A**, Münter S, Nagy Z, Faulhaber HD, Schuster H, Luft FC. Heritability analysis of lipids and three gene loci in twins link the macrophage scavenger receptor to HDL cholesterol concentrations. Arteriosclerosis Thrombosis and Vascular Biology,17, 1997, 2054-2060.

Busjahn A, Voss A, Knoblauch H, Knoblauch M, Jeschke E, Wessel N, Bohlender J, McCarron J, Faulhaber HD, Schuster H, Dietz R, Luft FC. Angiotensin-converting enzyme and angiotensinogen gene polymorphisms and heart rate variability in twins. Am J Cardiol,81, 1998, 755-760.

Pfeufer A, **Busjahn A**, Vergopoulos A, Knoblauch H, Urata H, Osterziel KJ, Menz M, Wienker TF, Faulhaber HD, Steinmetz A, Schuster H, Dietz R, Luft FC. Chymase gene locus is not associated with myocardial infarction and is not linked to heart size or blood pressure. Am J Cardiol,82, 1998, 979-981.

Busjahn A, Knoblauch H, Faulhaber HD, Boeckel T, Rosenthal M, Uhlmann R, Hoehe M, Schuster H, Luft FC. The QT interval is linked to two long-qt syndrome loci in normal subjects. Circulation, 99, 1999, 3161-3164.

Busjahn A, Faulhaber HD, Freier K, Luft FC. Genetic and environmental influences on coping styles: A twin study. Psychosom Med, 61, 1999, 469-475.

Busjahn A, Aydin A, v.Treuenfels N, Faulhaber HD, Gohlke HR, Knoblauch H, Schuster H, Luft FC. Linkage but lack of association for blood pressure and the α-adducin locus in normotensive twins. J Hypertens,17, 1999, 1437-1441.

Knoblauch H, **Busjahn A**, Müller-Myhsok B, Faulhaber HD, Schuster H, Uhlmann R, Luft FC. Peroxisome proliferator activated receptor γ gene locus is related to body mass index and lipid values in normal subjects. Arteriosclerosis Thrombosis And Vascular Biology, 19 (12), 1999, 2940-2944.

Nagy Z, **Busjahn A**, Bähring S, Faulhaber HD, Gohlke HR, Knoblauch H, Rosenthal M, Müller-Myhsok B, Schuster H, and Luft FC. Quantitative trait loci for blood pressure exist near the IGF-1, the liddle syndrome, the angiotensin II-receptor gene and the renin loci in man. JASN,10, 1999, 1709-1716.

Busjahn A, Li GH, Faulhaber HD, Rosenthal M, Becker A, Jeschke E, Schuster H, Timmermann B, Hoehe MR, Luft FC. β-2 adrenergic receptor gene variations, blood pressure, and heart size in normal twins. Hypertension, 35 (2), 2000, 555-560.

Knoblauch H, Müller-Myhsok B, **Busjahn A**, Ben Avi L, Bähring S, Baron H, Heath SC, Uhlmann R, Faulhaber HD, Shpitzen S, Aydin A, Reshef A, Rosenthal M, Eliav O, Mühl A, Lowe A, Schurr D, Harats, D Jeschke E, Friedlander Y, Schuster H, Luft FC, Leitersdorf E. A cholesterol-lowering gene maps to chromosome 13q. Am J Hum Gen, 66 (1), 2000, 157-166.

Busjahn A, Knoblauch H, Faulhaber HD, Aydin A, Uhlmann R, Tuomilehto J, Kaprio J, Jedrusik P, Januszewicz A, Strelau J, Schuster H, Luft FC, Müller-Myhsok B. A region on chromosome 3 is linked to dizygotic twinning. Nat Genet, 26, 2000, 398-399.

Tank J, Jordan J, Diedrich A, Stoffels M, Franke G, Faulhaber HD, Luft FC, **Busjahn A**. Genetic Influences on Baroreflex Function in Normal Twins. Hypertens, 37, 2001, 907-910.

Li GH, Faulhaber HD, Rosenthal M, Schuster H, Jordan J, Timmermann B, Hoehe MR, Luft FC, **Busjahn A**. β-2 adrenergic receptor gene variations and blood pressure under stress in normal twins. Psychophysiology (in press).

Busjahn A, Freier K, Faulhaber HD, Li GH, Rosenthal M, Timmermann B, Schuster H, Hoehe MR, Luft FC. β-2 Adrenergic Receptor Gene Variations and Coping Styles In Normal Twins. Behav Genet (subm.).

Inhaltsverzeichnis

Abkürzungsverzeichnis

a^2	additive genetische Einflüsse
BD	Blutdruck
BMI	Bodymass-Index
BRS	Baroreflex-Sensitivität
c^2	gemeinsame Umweltfaktoren (*common environment*)
d^2	nicht-additive genetische Einflüsse (*dominance*)
df	Freiheitsgrade (*degrees of freedom*)
DZ	dizygote Zwillinge
e^2	spezifische (ungeteilte) Umwelteinflüsse (*environment*)
EKG	Elektrokardiogramm
IBD	Identity by Descent
MZ	monozygote Zwillinge
p	Irrtumswahrscheinlichkeit / Signifikanzniveau
PCR	Polymerase-Kettenreaktion
QTL	Quantitative Trait Locus
SD	Standardabweichung (*standard deviation*)
SNP	Single Nucleotide Polymorphismus

1 Einleitung und Fragestellung

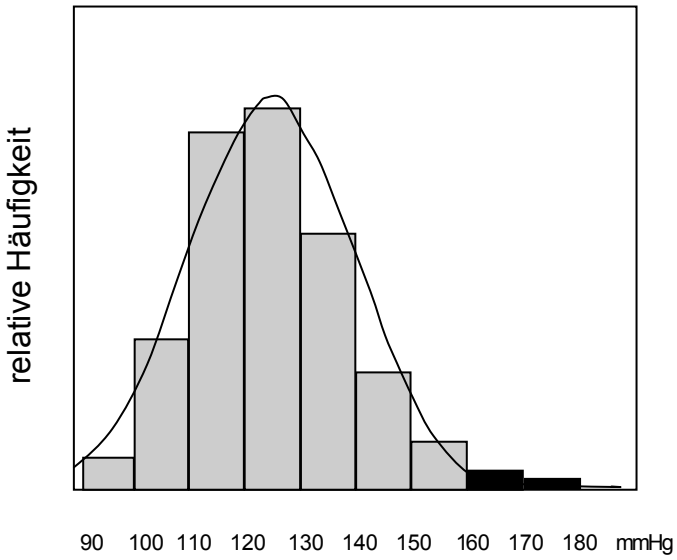
1.1 Genetische Einflüsse bei komplexen Erkrankungen

Mit Abschluss der Sequenzierphase des Humangenom-Projektes werden weitgehende Erwartungen an den Einfluß der Genetik auf das klinische Handeln verbunden. Die Kenntnis der Sequenzen allein ist jedoch nur ein erster, wenn auch wichtiger Schritt in Richtung einer molekulargenetisch fundierten Medizin. Funktionsbeschreibungen der Gene, Aufklärung der genetischen Variabilität einschließlich ihrer funktionellen Bedeutung sowie multivariate Analysen von genetischen und Umweltfaktoren sind notwendige weitere Schritte auf dem Weg.

In der bisherigen Geschichte der medizinischen Genetik steht den bemerkenswerten Erfolgen bei der Aufklärung seltener monogener Erkrankungen das weitgehende Fehlen von Erfolgen bei komplexen Erkrankungen mit weiter Verbreitung und entsprechender gesundheitspolitischer Bedeutung gegenüber. Die Ursachen hierfür sind vielfältig. Bereits durch die Beteiligung mehrerer Gene am pathogenen Prozeß wird die Wahrscheinlichkeit einer Genlokalisierung verringert. Dies gilt insbesondere, wenn die einzelnen Genpolymorphismen weder hinreichend noch notwendig für die Erkrankung sind, und wenn zwischen den Genen Wechselwirkungen bestehen. Die bei der Untersuchung verschiedener monogener Erkrankungen auftretenden Probleme der Heterogenität und eingeschränkten Penetranz verweisen auf die besonderen Schwierigkeiten beim Übergang zu komplexen Erkrankungen.

Die Krankheitsdiagnose (und somit Phänotypdefinition) bei Krankheiten wie der Hypertonie oder den Lipidstoffwechselstörungen beruht auf einer durch Konventionen festgelegten Schwellwertdefinition für einen quantitativen Messwert. Es besteht nicht notwendigerweise ein funktioneller oder struktureller Unterschied zwischen Patienten und gesunden Kontrollprobanden. Die Reduktion des Messwertes auf eine binäre qualitative Diagnose ist notwendigerweise mit einem Informationsverlust verbunden. Geringe Phänotypdifferenzen um den Schwellwert werden verstärkt, während große Differenzen innerhalb der beiden Gruppen nivelliert werden (Abbildung 1). Qualitativ definierte klinische Endpunkte wie Schlaganfall oder Herzinfarkt können als Folge einer Anfälligkeit (*Susceptibility*) mit quantitativer Verteilung betrachtet werden.

Abbildung 1: Verteilung des systolischen Blutdrucks (gemessen im Sitzen) in der Normalbevölkerung, der Diagnosebereich Hypertonie (>160mmHg) ist schwarz gekennzeichnet.



Veränderungen in verschiedenen Regulationssystemen können zu gleichen Verschiebungen in klinischen Messwerten führen. Auch innerhalb eines Regulationssystems wie dem Renin-Angiotensin-Aldosteron-System, dem adrenergen System oder der renalen Regulation der Salzausscheidung ist aufgrund der Vielzahl involvierter Genprodukte eine Heterogenität genetischer Einflüsse wahrscheinlich.

Zusätzlich wird die Untersuchung der Genetik komplexer Erkrankungen durch Umwelteinflüsse sowie Gen-Umwelt Interaktionen erschwert. Durch die Spezifik der menschlichen Umwelt in ihrer Komplexität von physischer Umwelt und sozialen Komponenten sind Untersuchungen am Tiermodell oder Zellsystem nur eingeschränkt aussagefähig.

1.2 Genetische Analysen bei intermediären Phänotypen

Ein möglicher Zugang zur Genetik komplexer Erkrankungen besteht in der Reduzierung der Komplexität durch Betrachtung einzelner zugrundeliegender physiologischer Größen, die auch als intermediäre Phänotypen bezeichnet werden. Dies bietet eine Reihe von Vorteilen. Zum einen sind physiologische Systeme durch Tier- und Humanexperimente oft genau charakterisiert und gestatten so die Definition von Kandidatengenen. Zum anderen umgeht die quantitative Erfassung von physiologischen Messgrößen das Problem des Informationsverlustes durch artifizielle Schwellwertdefinitionen.

Die Untersuchung der Funktion setzt nicht notwendigerweise pathologische Stichproben voraus. Nach Eintritt einer Erkrankung treten eine Reihe sekundärer Veränderungen auf, die nicht mit der Krankheitsentstehung assoziiert sind. Die Untersuchung physiologischer Variationen im Normalbereich umgeht diese konfundierenden Einflüsse.

Die Methoden der klassischen Humangenetik beruhen auf qualitativen Phänotypen und sind daher nicht einfach auf die Analyse quantitativer Messwerte übertragbar. Traditionell wurden analytische Methoden für quantitative Merkmale eher im Rahmen verhaltensgenetischer Analysen entwickelt und angewendet.

2 Methodik

2.1 Stichprobe

Alle im Folgenden dargestellten Untersuchungen wurden an einer kontinuierlich erweiterten Stichprobe von monozygoten (MZ) und dizygoten (DZ) Zwillingen durchgeführt. Die Rekrutierung erfolgte im Wesentlichen durch Anfragen an Teilnehmer einer Zwillingsstudie zu Persönlichkeitsmerkmalen an der Universität Bielefeld (1). Die Rekrutierung der dortigen Stichprobe beruhte auf Zeitungsanzeigen, redaktionellen Zeitschriftenbeiträgen sowie einer Zusammenarbeit mit Zwillingsclubs. Die Teilnehmer kommen aus allen Teilen Deutschlands. Innerhalb dieser Stichprobe von mehr als 2000 Zwillingspaaren wurde schriftlich nach der Bereitschaft zur Teilnahme an medizinischen Untersuchungen gefragt. Gegenwärtig sind 220 Zwillingspaare in die Untersuchung einbezogen (Tabelle 1).

Tabelle 1: Demographische Angaben zur Stichprobe.

	MZ	DZ	
N	244	200	
Alter (Jahre)	34±15	34±13	
Geschlecht M/W	80/164	60/140	
Größe, cm	169±9	170±9	
Gewicht, kg	67±13	71±14	
BMI, kg/m^2	23±4	24±4	

Die Zygotiebestimmung erfolgte mit genetischen Markern (2). Das mittlere Alter beträgt 35 Jahre, 32% der Teilnehmer sind männlich, monozygote Zwillinge sind mit 55% überrepräsentiert. Der relativ geringe Anteil an männlichen Teilnehmern und dizygoten Zwillingen ist ein typisches Phänomen bei Zwillingsuntersuchungen mit Rekrutierung durch öffentliche Aufrufe.

Für die Untersuchung von dizygoten Zwillingen anstelle von Geschwisterpaaren gibt es mehrere Argumente: die Altersübereinstimmung, die Übereinstimmung für intrauterine vorgeburtliche Einflüsse, die größere Übereinstimmung für Umweltfaktoren durch den größeren Anteil gemeinsam verbrachter Zeit, die gleiche Stellung innerhalb der Geschwisterreihe, die geringe Wahrscheinlichkeit falscher Vaterschaften für ein Paarmitglied und nicht zuletzt die größere Bereitschaft zur Teilnahme an genetischen Studien durch den bewussten Sonderstatus als Zwilling.

2.2 Phänotypisierung

Alle Phänotypanalysen erfolgten an der Franz Volhard Klinik. Die Probanden wurden zu einer etwa sechsstündigen Untersuchung in die Klinik eingeladen. Sie trafen gegen 08:00 Uhr nüchtern zur Untersuchung ein. Das Untersuchungsprogramm beinhaltete eine klinische Grunduntersuchung. Eingeschlossen waren eine Erhebung von Eigenanamnese und Familienanamnese zu Hypertonie, Fettstoffwechselstörungen, Herzinfarkt, Schlaganfall sowie sonstigen chronischen Erkrankungen. Es erfolgte eine Bestimmung von Körpergröße, Gewicht, Bauch- und Hüftumfang. Der Blutdruck wurde im Sitzen, Stehen und Liegen mittels Sphygmomanometer gemessen, die Herzfrequenz wurde manuell gemessen. Weiterhin erfolgte eine Untersuchung des Herzens mittels Ultraschall, dabei wurden sowohl morphologische Größen (z.B. Septumdicke, Hinterwanddicke, Herzvolumen) als auch funktionelle Parameter (Herzzeitvolumen) bestimmt. Eine EKG-Ableitung erfolgte auf verschiedene Weise, es wurde das in der Klinik übliche Ruhe-EKG abgeleitet und elektronisch ausgewertet, weiterhin erfolgte die Ableitung eines hochverstärkten EKG über 30 Minuten sowie eine Holter-EKG-Aufzeichnung über den gesamten Untersuchungsablauf.

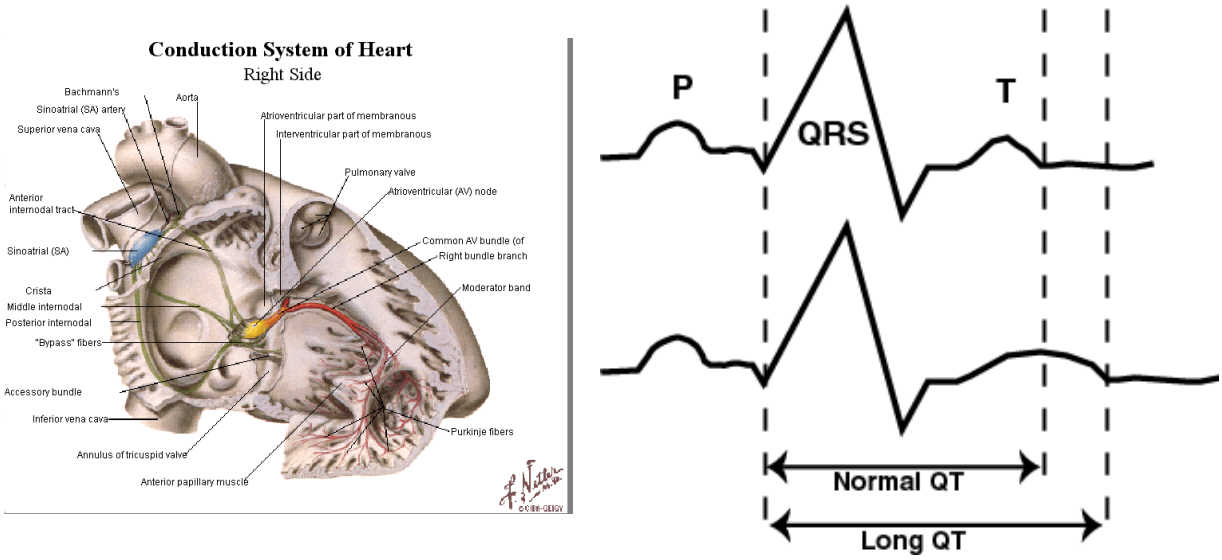
Weiterer wesentlicher Bestandteil der Untersuchung war die Messung der Kreislaufreaktionen auf psychische und physische Beanspruchung. Hierzu wurde ein spezieller Stresstest entwickelt. Der Test beinhaltet 5 verschiedene Anforderungen von 3 Minuten (Test 1 .. 3) bzw. 2 Minuten (Test 4, 5) Dauer:

1. STROOP-Test: Bei diesem Test wurden dem Probanden auf einem Bildschirm aufeinanderfolgend die Worte „rot“, „blau“, „grün“ und „gelb“ angezeigt, die Worte sind farbig dargestellt, Wort und Farbe sind stets diskordant. Die Aufgabe besteht in der Benennung der Farben. Durch den hochautomatisierten Impuls, Worte unabhängig von Schriftart und –farbe zu lesen, erfordert die Aufgabe hohe Konzentration. Eine zusätzliche Belastung erfolgte durch eine akustische Rückmeldung bei langsamen Reaktionszeiten, der Schwellwert wurde individuell ständig angepasst.
2. Kopfrechenaufgaben: Auf dem Bildschirm wurden Multiplikationsaufgaben dargestellt, der Schwierigkeitsgrad wurde fortlaufend individuell angepasst.
3. Psychomotorisches Tracking: Die Aufgabe bestand in der Steuerung eines Objektes auf dem Computerschirm durch einen Raum voller Hindernisse, die Schwierigkeit konnte durch die Probanden selbst angepasst werden.
4. Hand-Grip Test: Ein Gummiball, verbunden mit einer Druckanzeige, musste durch die Probanden kurzzeitig mit maximaler Kraft gedrückt werden. Auf der Anzeige wurde ein Drittel dieser Maximalkraft markiert, die Probanden mussten diese Kraft für zwei Minuten konstant beibehalten.
5. Cold-Pressor Test: Die Probanden mussten die linke Hand für zwei Minuten in 4° kaltes Wasser tauchen.

Die Ableitung physiologischer Parameter erfolgte während des gesamten Tests sowie während fünfminütiger Ruhephasen vor und nach dem Test. Der Blutdruck wurde kontinuierlich an einem Finger der rechten Hand mittels Finapres (Ohmeda, Louisville, CO, USA) gemessen sowie diskontinuierlich im Minutenabstand am linken Oberarm mittels Dinamap (Dinamap, Tampa, FL, USA). Auf Grund der eingeschränkten Validität der Finapresmethode für das absolute Blutdruckniveau wurden die Werte der kontinuierlichen Messung nur für die Berechnung der Blutdruckdifferenzen zwischen den Situationen verwendet. Eine EKG-Ableitung erfolgte mittels dreier Elektroden an beiden Beinen und dem rechten Unterarm, zusätzlich wurde die Atmung mittels Atemgürtel erfasst. Die Aufzeichnung von EKG, Atmung und analogem Blutdruckverlauf erfolgte mittels Analog-Digital-Wandler auf einem Computer (ParOn, Berlin, D).

Zur Untersuchung der Reizleitung im Herzen (Abbildung 2a) wurde aus dem EKG die QT-Zeit bestimmt. Hintergrund dieser Untersuchung ist das Vorliegen monogen bedingter Formen von verlängerter Reizleitungszeit (Abbildung 2b), des Long-QT Syndroms, bei denen ursächliche Gene bzw. Genloci bekannt sind.

Abbildung 2: Reizleitungssystem des Herzens (a, links) und schematische Darstellung eines EKG bei Vorliegen einer verlängerten Reizleitungszeit (b, rechts).



Zur Erfassung der Herzfrequenzvariabilität wurde aus der Langzeitaufzeichnung des EKG (Avionics Stratascan, Del Mar Avionics, Irvine, USA) das 30-Minutenintervall mit der geringsten Anzahl Artefakte ausgewählt. Die Schlagintervalle wurden automatisch bestimmt und über das Intervall gemittelt. Zur Bestimmung der Variabilität im Zeit- und Frequenzbereich wurden folgende Parameter berechnet:

1. Standardabweichung der Schlagintervalle über das Messintervall (SDNN)
2. Wurzel aus den gemittelten quadrierten Differenzen aufeinanderfolgender Schlagintervalle (RMSSD)
3. Powerspektralanalyse mittels Fourieranalyse im Frequenzbereich 0 – 0.4 Hz (P), 0 – 0.0033 Hz (ULF), 0.0044 – 0.04 Hz (VLF), 0.04 – 0.15 Hz (LF), sowie der normalisierten Power im Bereich von 0 – 0.15 Hz / P (HF)
4. Prozentsatz von Schlagintervallen mit Differenzen kleiner 10 ms (PNNL10), kleiner 20 ms (PNNL20) und grösser 100ms (PNN100)

Basierend auf den Daten der Ruhephase des Stresstests erfolgte eine Berechnung verschiedener Masse der Baroreflex-Sensitivität (BRS). Dazu erfolgte eine Analog-Digitalwandlung der physiologischen Parameter (EKG 1 kHz, BD 100 Hz). Die Detektion von R-Zacken, systolischem und diastolischem Blutdruck erfolgte offline mittels PV-wave Software (VisualNumerics, Houston, TX, USA).

Folgende Parameter wurden berechnet:

1. Der Anstiegswinkel des Spontanbaroreflexes (*spontaneous baroreflex slope*) wurde bestimmt als der Anstiegswinkel der linearen Regression zwischen systolischem Blutdruck und R-R-Intervall im EKG mittels Sequenztechnik. Sequenzen mit mindestens drei aufeinanderfolgenden Intervallen von mindestens 0.5 mmHg Blutdruckunterschied und 5 ms R-R-Interval-Veränderung wurden analysiert. Nur Intervalle mit Korrelationskoeffizienten von >0.85 wurden als signifikant betrachtet. BRS wurde bestimmt als Mittelwert aller signifikanten Regressionsgeraden, dabei wurden ansteigende Sequenzen (BRS+) und absteigende Sequenzen (BRS-) getrennt betrachtet.
2. Baroreflex-Kreuzspektralanalyse. Die Powerspektralanalyse ist eine bewährte Methode zur Analyse zeitlicher Fluktuationen in Herz-Kreislaufparametern (z.B. Herzfrequenz). Für die Analyse der Beziehung zwischen verschiedenen Parametern und ihren verschiedenen Rhythmen dient in vergleichbarer Weise die Kreuzspektralanalyse. Die Analysen erfolgten für die Kreuzspektren von systolischem Blutdruck und R-R-Intervallen (Segmentlänge 256 s, Zahl der Messpunkte 1024, Auflösung 0.004 Hz). Der Baroreflex wurde berechnet als Mittelwert der Transferfunktion im unteren (BRSlow) und oberen Frequenzbereich (BRShigh). Als signifikant wurden Ergebnisse einbezogen, wenn die Kohärenz im jeweiligen Frequenzbereich >0,8 betrug.

Im Anschluss an die Ableitung des hochverstärkten EKG und der damit verbundenen 30-minütigen Ruhephase im Liegen erfolgte eine Blutabnahme. Aus der Blutprobe erfolgte die Extraktion genomischer DNA für die molekulargenetischen Analysen, die Bestimmung von Lipiden (HDL-Cholesterin, LDL-Cholesterin, Triglyceride), Noradrenalin, Adrenalin, Angiotensin-Konversionsenzym sowie Angiotensinogen.

Zur Untersuchung der Stressbewältigung untersuchten wir 19 Copingstrategien, erfasst mit dem Stressverarbeitungs-Fragebogen SVF von Jahnke et al. (3). Beispiel-Fragen für die einzelnen Skalen sind in Tabelle 2 dargestellt.

Tabelle 2: Stressverarbeitungs-Fragebogen mit Einzelskalen und Beispiel-Fragen. Die Beantwortung erfolgt auf einer Skala von 0-4.

Alle Fragen beginnen mit: "Wenn ich durch irgendetwas oder irgendjemanden beeinträchtigt, innerlich erregt oder aus dem Gleichgewicht gebracht worden bin,..."

Skala 1:	**Bagatellisierung**: ...sage ich mir, es geht schon wieder alles in Ordnung
Skala 2:	**Herunterspielen durch Vergleich mit anderen**: ...nehme ich das leichter als andere in der gleichen Situation
Skala 3:	**Schuldabwehr**: ...denke ich, ich habe die Situation nicht zu verantworten
Skala 4:	**Ablenkung von Situationen**: ...lenke ich mich irgendwie ab
Skala 5:	**Ersatzbefriedigung**: ...erfülle ich mir einen lang ersehnten Wunsch
Skala 6:	**Suche nach Selbstbestätigung**: ...verschaffe ich mir Anerkennung auf anderen Gebieten
Skala 7:	**Situationskontrollversuche**: ...mache ich einen Plan, wie ich die Schwierigkeiten aus dem Weg räumen kann
Skala 8:	**Reaktionskontrollversuche**: ...sage ich mir, du darfst die Fassung nicht verlieren
Skala 9:	**Positive Selbstinstruktion**: ...sage ich mir, du kannst damit fertig werden
Skala 10:	**Bedürfnis nach sozialer Unterstützung**: ...versuche ich, mit irgendjemandem über das Problem zu sprechen
Skala 11:	**Vermeidungstendenz**: ...nehme ich mir vor, solchen Situationen in der Zukunft aus dem Weg zu gehen
Skala 12:	**Fluchttendenz**: ...neige ich dazu, die Flucht zu ergreifen
Skala 13:	**Soziale Abkapselung**: ...meide ich die Menschen
Skala 14:	**Gedankliche Weiterbeschäftigung**: ...beschäftigt mich die Situation hinterher noch lange
Skala 15:	**Resignation**: ...neige ich dazu, zu resignieren
Skala 16:	**Selbstbemitleidung**: ...frage ich mich, warum das gerade mir passieren musste
Skala 17:	**Selbstbeschuldigung**: ...mache ich mir Vorwürfe
Skala 18:	**Aggression**: ...werde ich ungehalten
Skala 19:	**Pharmakaeinnahme**: ...neige ich dazu, irgendwelche Medikamente zu nehmen

Zur Reduktion der Variablenzahl wurden zusätzlich zu den 19 Skalen Sekundärfaktoren berechnet, die die Grundlage der genetischen Analysen bildeten. Die

Einzelskalen werden nur im Sinne einer Hypothesengeneration dargestellt. Die vier Faktoren lassen sich folgendermassen beschreiben:

SVF1. **Verteidigung** (Bagatellisierung, Herunterspielen durch Vergleich, Schuldabwehr, Positive Selbstinstruktion)

SVF2. **Emotionales Coping** (Fluchttendenz, Soziale Abkapselung, Gedankliche Weiterbeschäftigung, Resignation, Selbstbeschuldigung, Aggression)

SVF3. **Ersatz** (Ersatzbefriedigung, Bedürfnis nach sozialer Unterstützung, Pharmakaeinnahme)

SVF4 **Aktives Coping** (Situationskontrollversuche, Reaktionskontrollversuche, Positive Selbstinstruktion, Vermeidungstendenz).

2.3 Genotypisierung

Die DNA-Extraktion erfolgte aus Leukozyten. Jedem Probanden wurden hierzu zweimal 10 ml Blut aus einer Vene entnommen. Bei der Genotypisierung wurden unterschiedliche Methoden verwendet, der Hauptunterschied betrifft die Analyse von Mikrosatelliten einerseits und Mutationen/Polymorphismen andererseits.

Für die Bestimmung der Zygotie sowie Kopplungsanalysen erfolgte die Typisierung von Mikrosatelliten. Dabei handelte es sich um hochpolymorphe Abschnitte der DNA mit mehrfacher Wiederholung kurzer Sequenzen, überwiegend aus zwei Basen (auch als *variable number of tandem repeats* VNTR bezeichnet). Zu einem geringeren Teil wurden auch Mikrosatelliten-Marker mit 3 oder 4 repetitiven Basen verwendet. Der Vorteil dieser Marker liegt in der besseren Alleldiskriminierung durch die größere Längendifferenz der einzelnen Allele. Details der einzelnen verwendeten Mikrosatelliten sind in den jeweiligen Publikationen angegeben. Der generelle Ablauf der Analysen wird im Folgenden beschrieben:

Die Genloci der Mikrosatelliten-Marker werden mit Hilfe der Polymerase-Kettenreaktion (PCR) amplifiziert und dabei mit spezifischen Farbstoffen markiert. Marker mit verschiedenen Farbstoffen lassen sich in einer PCR zusammenfassen. In Abhängigkeit von der Größe der Allele lassen sich auch mehrere Marker mit dem gleichen Farbstoff markieren und gemeinsam analysieren.

Die Analyse der Allelgrößen erfolgte auf DNA-Sequencern der Firma Applied Biosystems (ABI, Foster City, CA, USA), es wurden sowohl gelbasierte Analysen auf

einem Sequencer 373 als auch kapillarbasierte Analysen auf einem Sequencer 377 durchgeführt. Die Auswertung der Genotypen erfolgte mittels Software (Genescan und Genotyper, ABI) mit manueller Kontrolle der Ergebnisse.

Der Insertions-/Deletionspolymorphismus im Gen des Angiotensin-Konversionsenzyms wurde mittels allelspezifischer PCR bestimmt (4, 5).

Als weitere Methode zur Detektion von Polymorphismen wurde der 5´ Nuclease-Assay (auch TaqMan genannt) eingesetzt (6). Wir verwendeten diese Methode zum Nachweis eines biallelischen Polymorphismus im α-Adducin Gen. Der 460 Trp Polymorphismus wurde im Zusammenhang mit essentieller Hypertonie bei italienischen und französischen Patienten untersucht (7). Der 5‘ Nuclease-Assay wurde für diesen Polymorphismus optimiert. Die Analyse einer einzelnen Probe (nach PCR) dauert im Durchschnitt 10 Sekunden.

Die PCR-Primer für die 460 Trp Mutation wurden wie folgt synthetisiert: 5´ -TCG TCC ACA CCT TAG TCT TCG A; 3´-GCA GCG GGA GAA GAC AAG AT. Die Fluoreszenzsonden bestehen aus einem Oligonucleotid, das sowohl mit einem Reporterfarbstoff als auch mit einem Quencher markiert ist. Beide Sonden besitzen den gleichen Quencher, aber jeweils spezifische Reporterfarbstoffe. Eine Sonde hybridisiert mit dem Wildtypallel, in diesem Fall 460 Gly, und die zweite Sonde mit dem Mutationsallel, 460 Trp. Die Sequenz der Wildtyp-Sonde war: 5´-TET - TCC ATT CTG CCC TTC CTC GGA AG-TAMRA; die Sequenz der Mutations-Sonde war: 5´-FAM- TTC CAT TCT GCC ATT CCT CGG AAG-TAMRA.

Beide Sonden werden gleichzeitig in der PCR eingesetzt. Sie werden anhand der Reporterfarbstoffe FAM (6-carboxy-fluorescein) und TET (6-carboxy-4, 7, 2´, 7´-tetrachloro-fluorescein) unterschieden. Eine Hybridisierung der FAM Sonde in der PCR Reaktion resultiert in einer Hydrolyse durch die 5´ Nuclease Aktivität der Taq Polymerase und der Freisetzung eines Fluoreszenzsignals. Die Hybridisierung der TET Sonde resultiert in gleicher Weise in Hydrolysierung und Freisetzung eines anderen Fluoreszenzsignals. Bei Nichtübereinstimmung von Sonde und DNA-Sequenz ist die 5‘ Nuclease Aktivität so ineffektiv, dass wenig oder keine Fluoreszenz freigesetzt wird. Bei heterozygoten Proben werden beide Fluoreszenzsignale mit verringerter Aktivität freigesetzt. Nach Abschluss der PCR-Reaktion werden die 96 Proben auf einem Tray in einem TaqMan 7700 Reader (Perkin Elmer) analysiert. Dabei wird für jede Probe die Fluoreszenzaktivität beider Reporterfarbstoffe bestimmt und einem Genotyp zugeordnet.

Der Nutzen dieser Methode liegt in der Allelbestimmung bekannter Polymorphismen bei großen Stichproben. Pro Tag können mehrere hundert Proben analysiert werden. Das System ist automatisiert und liefert die Ergebnisse in digitaler Form zur Weiterverarbeitung. Die PCR Reaktion und die Genotypisierung sind in einem Schritt zusammengefasst.

2.4 Heritabilitätsberechnung bei Zwillingen

Heritabilität bezeichnet den relativen Anteil der Merkmalsvariation, der durch genetische Variation bedingt ist. Angaben zur Heritabilität sind somit nur für die Population gültig, aus der die jeweilige Stichprobe gezogen wurde. Populationsunterschiede können sowohl die Merkmalsvariabilität als auch die zugrundeliegenden genetischen und Umweltvariationen betreffen. Auch zeitlich sind Heritabilitätsangaben eingeschränkt, insbesondere durch mögliche Veränderungen der Umweltvariation. Bei der Bestimmung der Heritabilität kann unterschieden werden zwischen additiv wirksamen genetischen Einflüssen und genetischen Faktoren mit Allel-Interaktion / Dominanz. Gelegentlich werden beide genetischen Einflüsse zusammengefasst (*broad-sense heritability*).

Grundlage der Berechnung der Heritabilität bildet der Vergleich der phänotypischen Übereinstimmung zwischen Gruppen mit unterschiedlichem Grad genetischer Übereinstimmung. Untersuchungsdesigns mit Vergleich zwischen Generationen (z.B. Eltern-Kind) können nur additive genetische Einflüsse testen, da zwischen den Generationen nur Übereinstimmung für jeweils 1 Allel besteht. Weiterhin sind solche Untersuchungen durch Alterseinflüsse auf den Phänotyp sowie eventuelle Umweltveränderungen zwischen den Generationen (z.B. Ernährung in Krisenzeiten) nur bedingt aussagefähig.

Die phänotypische Übereinstimmung zwischen Geschwistern kann sowohl durch genetische Übereinstimmung als auch durch familiäre Umweltfaktoren bedingt sein und gestattet somit keine valide Abschätzung der Heritabilität.

Eine Möglichkeit der Quantifizierung genetischer Einflüsse besteht im Vergleich monozygoter und dizygoter Zwillingspaare. Beide Arten von Zwillingspaaren teilen in gleicher Weise familiäre Umweltfaktoren. Sie unterscheiden sich aber im Grad der genetischen Übereinstimmung. Monozygote Zwillinge (MZ) sind – mit der Ausnahme postzygotischer Mutationen (8) genetisch identisch, dizygote Zwillinge

(DZ) haben eine genetische Übereinstimmung von durchschnittlich 50%. Eine größere Übereinstimmung bei MZ im Vergleich zu DZ verweist somit auf genetische Einflüsse.

Die Bestimmung der Heritabilität an Zwillingen ist an bestimmte Voraussetzungen gebunden. Von zentraler Bedeutung sind die Annahme der genetischen Identität von MZ sowie die Annahme, dass die Umwelteinflüsse bei MZ nicht stärker übereinstimmen als bei DZ (9). Insbesondere systematische genetische Differenzen bei MZ als Grundlage der Trennung von Zellen innerhalb der ersten 10-14 Tage der Schwangerschaft könnte zu einer Unterschätzung genetischer Einflüsse führen, solche Differenzen konnten bisher jedoch nicht nachgewiesen werden. Die Annahme gleicher Umweltübereinstimmung lässt sich auftrennen in prä- und postnatale Umweltfaktoren. Für die Zeit nach der Geburt wurden zahlreiche Untersuchungen durchgeführt, die auf der Analyse von Zwillingspaaren mit falsch zugeschriebener Zygotie oder auch MZ mit verschiedenem Grad äußerer Übereinstimmung beruhen. In diesen Untersuchungen konnte die Annahme gleicher Umweltfaktoren bestätigt werden. Für die pränatale Umwelt gilt dies nicht in gleicher Weise. MZ unterscheiden sich hier im Grad der gemeinsamen Umweltfaktoren: MZ können sich als monochorionisch/monoamniotische, monochorionisch/diamniotische oder dichorionosch/diamniotische Embryonen entwickeln, während DZ dichorionosch/diamniotische Embryonen darstellen. Ausgehend von der Theorie des ‚*Fetal Programming*' untersuchte Phillips (10,11) die Hypothese, dass eine stärkere Übereinstimmung für pränatale Einflüsse bei monochorionischen MZ zu einer Überschätzung genetischer Einflüsse führt, während andererseits die gemeinsame Versorgung mit Nährstoffen zu einer Konkurrenzsituation führen kann, die zu phänotypischen Unterschieden führt und somit dem Chorioneffekt entgegenwirkt. Zur Bestimmung der Stärke des Chorioneffekts bei bekanntem Choriontyp wurden mathematische Modelle entwickelt (12), allerdings sind die notwendigen Angaben nicht immer mit ausreichender Sicherheit zu erhalten.

Insgesamt ist also die Heritabilitätsbestimmung an Zwillingen wie jede andere Analysemethode kein perfektes Abbild der biologischen Wirklichkeit, sie stellt aber die beste bekannte Annäherung dar. Während Verletzungen der Voraussetzungen im Einzelfall zu einer Verfälschung der Schätzwerte führen kann, trifft diese Ein-

schränkung für die Ergebnisse der Analyse einzelner Gene mittels Kopplungs- und Assoziationsanalysen, die später erläutert werden, nicht zu.

Zur Quantifizierung genetischer Einflüsse mittels Zwillingsuntersuchungen erfolgt eine Varianzkomponenten-Analyse (13). Die Varianz eines Merkmals in der Population wird definiert durch die Varianz additiver genetischer Faktoren (Var_{addGen}), die Varianz nicht-additiver (dominanter) genetischer Faktoren (Var_{domGen}) sowie die Varianz der Umweltfaktoren. Diese lassen sich weiter unterteilen in Umweltfaktoren, die beide Zwillinge teilen ($Var_{sharedEnv}$) sowie unkorrelierte Umweltfaktoren (Var_{Env}):

$$Var = Var_{addGen} + Var_{domGen} + Var_{sharedEnv} + Var_{Env}$$

Die phänotypische Übereinstimmung (Kovarianz) bei Zwillingen ist definiert durch:

$$Covar_{MZ} = Var_{addGen} + Var_{domGen} + Var_{sharedEnv}$$

$$Covar_{DZ} = 0.5\ Var_{addGen} + 0.25\ Var_{domGen} + Var_{sharedEnv}$$

Durch Bestimmung von Varianz und Kovarianz lassen sich die Varianzanteile von genetischen und Umweltfaktoren abschätzen. Dazu werden in einem Modellierungsverfahren mittels numerischer Optimierung Parameter für die Faktoren bestimmt, bei denen empirische Daten und Modellvorhersage bestmöglich übereinstimmen. Die Güte des Modells sowie die Signifikanz der einzelnen Faktoren lassen sich über einen χ^2-Test bestimmen. Dabei werden einzelne Faktoren aus dem Modell gestrichen, die Güte der Modellanpassung wird zwischen reduziertem und vollständigem Modell verglichen.

Nicht-additive genetische Faktoren vergrößern den Unterschied zwischen der Kovarianz von MZ und DZ ($Covar_{MZ} > 2 * Covar_{DZ}$), gemeinsame Umweltfaktoren dagegen verringern die Differenz der Kovarianz ($Covar_{MZ} < 2 * Covar_{DZ}$). Daher lassen sich beide Faktoren nicht simultan bestimmen. Nur der jeweils stärkere Faktor lässt sich empirisch nachweisen.

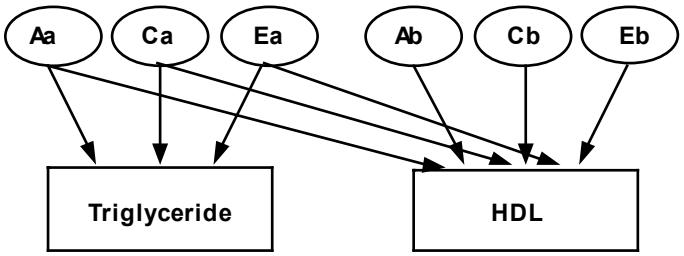
Zur Durchführung der Analysen wurden die Programme LISREL (14) und Mx (15) benutzt.

Theoretisch sind auch die Modellierung von weiteren nicht-additiven genetischen Einflüssen (z.B. Epistase) sowie von Wechselwirkungen möglich. Da solche Analysen im Rahmen der hier dargestellten Studien nicht durchgeführt wurden, wird auf die theoretischen Grundlagen nicht weiter eingegangen.

2.5 Multivariate Analysen zur genetischen Korrelation zwischen Phänotypen

Korrelation zwischen verschiedenen Phänotypen (z.B. Blutdruck und Körpergewicht) können sowohl durch gemeinsame genetische Faktoren bedingt sein (genetische Korrelation) als auch durch Umweltfaktoren bedingt werden. Zwillingsstudien gestatten eine Quantifizierung der genetischen Korrelation zwischen zwei oder mehr quantitativen Merkmalen. Die Analyse kann zum einen durch die Quantifizierung spezifischer und gemeinsamer genetischer Einflüsse erfolgen (Cholesky-Dekomposition, Abbildung 3), zum anderen lassen sich die genetischen Korrelationen direkt berechnen.

Abbildung 3: Bivariate Analyse als einfachste Form einer Cholesky-Dekomposition, hier Analyse gemeinsamer und spezifischer Faktoren für Triglyceride und HDL-Cholesterol. Aa bezeichnet gemeinsame additive genetische Faktoren, Ab spezifische additive genetische Faktoren für einen Phänotyp. Ca und Cb bzw. Ea und Eb bezeichnet in gleicher Weise gemeinsame und spezifische Faktoren der familiären und sonstigen Umwelt.



Die Bedeutung der Analyse genetischer Korrelationen liegt zum einen in der Aufklärung kausaler Wirkungszusammenhänge in komplexen regulatorischen Systemen bzw. dem Ausschluss nichtkausaler Korrelationen zwischen Messwerten aufgrund externer Faktoren oder zeitlicher Zusammenhänge. Zum anderen erhöht die Analyse mehrerer Phänotypen mit genetischer Korrelation (bzw. die Einbeziehung verschiedener Messverfahren für einen Parameter) die Power für die Lokalisierung von Genen (16).

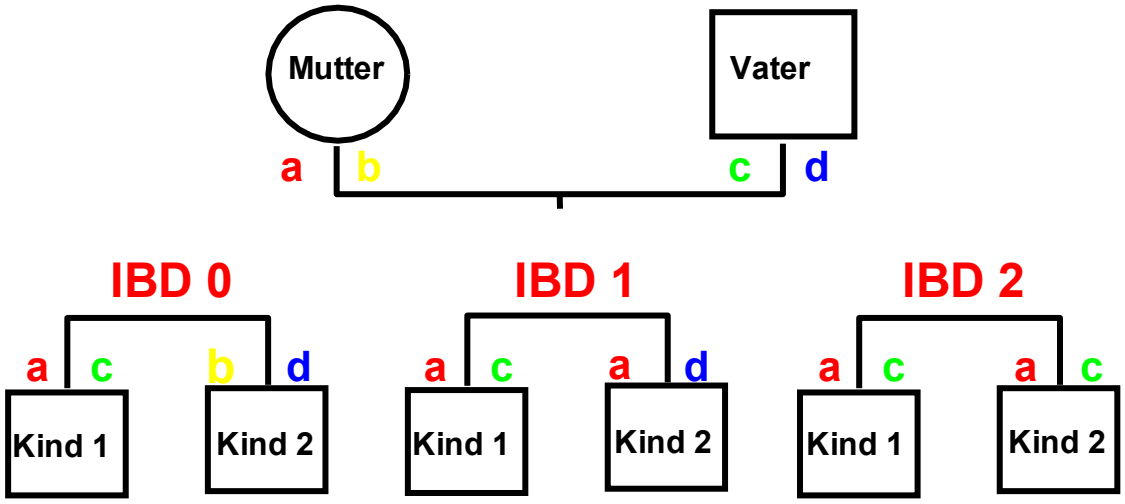
2.6 Kopplungsanalysen mit quantitativen Messwerten

Die Kopplungsanalyse von Genloci und quantitativen Messwerten kann sowohl mittels parametrischen als auch parameterfreien Verfahren erfolgen. Alle hier berichteten Studien beruhen auf nichtparametrischen Geschwisterpaar-Analysen.

Grundlage aller im Folgenden beschriebenen Analysen ist die Berechnung der genetischen Identität innerhalb der Geschwisterpaare. Für jeden beliebigen Genlokus erbt ein Kind von den beiden mütterlichen Allelen eines zufällig, das gleiche gilt für die väterlichen Allele. Da die Übertragung der elterlichen Allele an jedes Kind unabhängig erfolgt, beträgt die Wahrscheinlichkeit, dass beide Geschwister identische Genvarianten geerbt haben (Identity by Descent, IBD2), 25%. Die Wahrscheinlichkeit, ein gemeinsames elterliches Allel zu tragen (IBD1), beträgt 50%, und die Wahrscheinlichkeit keiner genetischen Übereinstimmung (IBD0) 25% (Abbildung 4).

Sind die elterlichen Genotypen bekannt und informativ (beide Eltern heterozygot für verschiedene Allele), lässt sich der IBD-Status der Kinder eindeutig bestimmen. Andernfalls lässt sich anhand der Allelfrequenzen die Wahrscheinlichkeit der genetischen Übereinstimmung berechnen. Diese Berechnungen erfolgten bei den dargestellten Analysen innerhalb der Programmpakete S.A.G.E. (17) und Mapmaker-Sibs (18).

Abbildung 4: Vergleich der Weitergabe elterlicher Allele an Geschwister (*Identity by Descent*)



Die generelle Annahme der verschiedenen Analysemethoden besteht darin, dass im Fall vorhandener Kopplung Paare mit größerer genetischer Übereinstimmung (IBD) auch für die entsprechenden Phänotypen eine Ähnlichkeit zeigen sollten. Die am weitesten verbreitete Analysemethode wurde von Haseman und Elston entwickelt (19) und beruht auf einer Regression zwischen dem IBD-Status (pi-hat, transformiert auf einer Skala von 0 bis 1) und der quadrierten Paardifferenz für

den Messwert. Eine signifikante negative Regression ist der Nachweis einer Kopplung zwischen Markergen(en) und einem funktionell relevanten Gen.

Der Nachteil dieser Analyse besteht im Informationsverlust durch die Reduzierung auf Paardifferenzen ohne Einbeziehung der Lage im Bezug auf den Populationsmittelwert. Weiterhin bietet das Programm S.A.G.E. keine Multipoint-Analysen mit simultaner Auswertung mehrerer Mikrosatelliten-Marker.

Eine alternative Analysemethode beruht auf der Analyse von Varianz-Kovarianz-Matrizen (20). Die Logik dieser Analyse besteht in einer Erweiterung der klassischen Zwillingsanalysen. In der Varianzdefinition wird der genetische Einfluß aufgespalten in Varianz bedingt durch den untersuchten Lokus (Var_{QTL})und Varianz des genetischen Hintergrundes (Var_{addGen}):

$$Var = Var_{QTL} + Var_{addGen} + Var_{Env}$$

Die erwartete Kovarianz ist abhängig vom Grad der genetischen Übereinstimmung:

$$Covar_{IBD0} = 0.5\ Var_{addGen}$$

$$Covar_{IBD1} = 0.5\ Var_{QTL} + 0.5\ Var_{addGen}$$

$$Covar_{IBD2} = Var_{QTL} + 0.5\ Var_{addGen}$$

In einer Modellanalyse werden anhand der empirischen Varianzen und Kovarianzen die Parameter für den relativen Einfluß von putativem QTL und genetischem Hintergrund bestimmt. Die Wahrscheinlichkeiten für die genetische Übereinstimmung für 0,1 und 2 Allele werden als Gewichtungsfaktoren für die drei Analysegruppen verwendet. Die Signifikanz des QTL-Effekts (Kopplung) wird durch Vergleich der Anpassungsgüte von Modellen mit und ohne QTL-Effekt getestet. Die Analysen wurden mit dem Programm Mx berechnet (15).

2.7 Assoziationsanalysen mit quantitativen Messwerten

Eine Beziehung zwischen Allelvarianten (A_1A_1, A_1A_2 und A_2A_2) und quantitativen Messwerten läßt sich durch varianzanalytischen Vergleich zwischen den drei Genotypen nachweisen. Unter der Annahme der Dominanz eines Allels kann der Vergleich als t-Test durchgeführt werden (A_1A_1/ A_1A_2 vs. A_2A_2). Assoziationsanalysen unterliegen der Gefahr falsch positiver Ergebnisse durch

Populationsstratifizierung, wenn sich die Stichprobe aus mehreren Populationen zusammensetzt, zwischen denen Unterschiede in den Allelfrequenzen sowie der Phänotypverteilung auftreten. Zur Vermeidung dieses potenziellen Risikos wurde für Fall-Kontrollstudien der Transmissions-Distortion-Test (TDT) entwickelt (21) und später auch für quantitative Phänotypen weiterentwickelt (22). Die TDT-Analysen benutzen die elterlichen Allele als Kontrollgruppe. Entscheidende Nachteile dieser Analysen bestehen in der dreifach erhöhten Anzahl durchzuführender Genotypisierungen sowie einer verringerten Power, da nur Probanden informativ sind, bei denen die Eltern für die Markerallele heterozygot sind. Für die Untersuchung von Probanden im höheren Lebensalter sind DNA-Proben der Eltern nicht oder nur schwer zu erhalten, dieses Problem ist aber im Rahmen unserer Untersuchungen von geringerer Bedeutung.

Alternativ zur Analyse der elterlichen Allele als Familienkontrolle läßt sich eine Populationsstratifizierung bei Assoziationen mit quantitativen Merkmalen auch durch Geschwisterpaar-Analysen nachweisen (20). Für zwei Allele A_1 und A_2 läßt sich der quantitative Alleleffekt a sowohl aus der Phänotypdifferenz innerhalb der Geschwisterpaare (z.B. $A_1A_1 – A_1A_2$) abschätzen, als auch aus der Differenz zwischen Geschwisterpaaren (z.B. $A_1A_1/A_1A_1 – A_1A_1/A_1A_2$). Der Alleleffekt für die verschiedenen Paartypen ist in Tabelle 3 dargestellt.

Tabelle 3: Alleleffekte in Abhängigkeit von den Genotypen von Geschwisterpaaren:

Geschwister1	Geschwister2	Alleleffekt 1	Alleleffekt 2	Alleleffekt auf Paarmittel	Alleleffekt auf Paardifferenz
A_1A_1	A_1A_1	a	a	a	0
A_1A_1	A_1A_2	a	0	a/2	a
A_1A_1	A_2A_2	a	-a	0	2a
A_1A_2	A_1A_1	0	a	a/2	-a
A_1A_2	A_1A_2	0	0	0	0
A_1A_2	A_2A_2	0	-a	-a/2	a
A_2A_2	A_1A_1	-a	a	0	-2a
A_2A_2	A_1A_2	-a	0	-a/2	-a
A_2A_2	A_2A_2	-a	-a	-a	0

Da beide Mitglieder eines Geschwisterpaares aus der gleichen Population stammen, ist die Differenz innerhalb der Paare nicht durch Populationsstratifizierung

beeinflusst und gestattet einen validen Signifikanztest. In Abwesenheit von Populationsstratifizierung sind die geschätzten Alleleffekte basierend auf Differenzen innerhalb und zwischen Paaren gleich und gestatten eine genauere Quantifizierung. Treten signifikante Populationseinflüsse auf, lassen sie sich aus der Differenz der beiden Schätzwerte quantitativ bestimmen. Der Test erfolgt durch eine Modellierung der Alleleffekte, basierend auf den Phänotypdifferenzen. Der Test auf Populationseinflüsse erfolgt durch den Vergleich der Anpassungsgüte zwischen zwei Modellen. Bei einem Modell erfolgt eine separate Abschätzung der Alleleffekte aus den Differenzen innerhalb und zwischen den Paaren. Im zweiten Modell werden beide Schätzwerte gleichgesetzt. Wenn beide Modelle keinen signifikanten Unterschied ergeben, wird der Schätzwert aus dem zweiten Modell auf Signifikanz getestet. Bei Auftreten signifikanter Populationseinflüsse erfolgt dieser Test nur für den Alleleffekt aus den Differenzen innerhalb der Paare. Die Signifikanz des Alleleffekts erfolgt ebenfalls durch den Vergleich der Güte der Modellanpassung zwischen Modellen mit und ohne Alleleffekt.

3 Darstellung einzelner Studienergebnisse

3.1 Heritabilitätsberechnungen und genetische Korrelationen

3.1.1 Kardiovaskuläre Parameter

Für den Ruheblutdruck wurden Heritabilitätswerte in verschiedenen Publikationen berichtet (23,24,25,26,27). Da diese Berechnungen auf unterschiedlichen Stichprobengrößen beruhen, werden an dieser Stelle nur die Ergebnisse der letzten Berechnungen dargestellt (26). Generell wird im Folgenden jeweils der Wert berichtet, der auf der größten Stichprobe basiert.

Die Blutdruckwerte der Zwillingsstichprobe zeigten keine Unterschiede zwischen monozygoten und dizygoten Zwillingen. Der Blutdruck im Liegen, Sitzen und Stehen wies signifikante genetische Einflüsse auf, die Heritabilitätswerte lagen zwischen 0,64 und 0,74 (Tabelle 4). Die zugrundeliegende Korrelation wird exemplarisch für den systolischen Blutdruck graphisch dargestellt (Abbildung 5).

Abbildung 5: Streudiagramm des systolischen Blutdrucks bei MZ und DZ

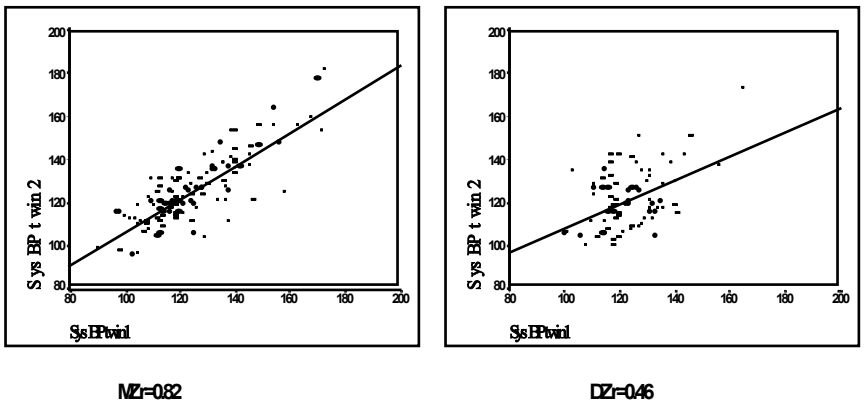


Tabelle 4: Blutdruck und Herzmorphologie (Mittelwert ± SD), Korrelationen (r) und Heritabilität (a^2)

Phänotyp	MZ	DZ	a^2 (r_{MZ}/r_{DZ})	
N	200	132		
systolischer BD liegend, mmHg	128±17	124±14	0.69 (0.69/0.31)	
diastolischer BD liegend, mmHg	71±12	71±11	0.66 (0.66/0.42)	
systolischer BD sitzend, mmHg	125±16	123±13	0.74 (0.74/0.38)	
diastolischer BD sitzend, mmHg	73±11	73±10	0.72 (0.72/0.51)	
systolischer BD stehend, mmHg	124±15	122±14	0.67 (0.66/0.48)	
diastolischer BD stehend, mmHg	80±10	79±10	0.64 (0.63/0.40)	
Herzhinterwanddicke, mm	8.7±1.6	8.6±1.6	0.48 (0.48/0.26)	
Septumdicke, mm	8.9±1.7	8.8±1.6	0.64 (0.64/0.37)	
linksventrikuläre Masse, mm^3	165±50	176±60	0.68 (0.68/0.27	

Neben den Blutdruckwerten im Stehen, Liegen und Sitzen, die mittels Sphygmomanometer erhoben wurden, liegen auch automatische Blutdruckmessungen mittels Dinamap (Blutdruckniveau) und Finapres (Blutdruckveränderung) aus dem Stresstest vor. Für das Blutdruckverhalten während der Ruhephase, dem Kopfrechnen und dem Cold-Pressor Test konnten genetische Einflüsse nachgewiesen werden. Auch die belastungsinduzierten Blutdruck- und Herzfrequenzanstiege (mit

Ausnahme des Anstiegs des systolischen Blutdrucks während des Kopfrechnens) zeigten signifikante genetische Einflüsse (Tabelle 5).

Tabelle 5: Blutdruckwerte während des Stresstests (Mittelwert ± SD), Korrelationen (r) und Heritabilität (a^2) (N: 200 MZ, 132 DZ).

Phänotyp	MZ	DZ	a^2 (r_{MZ}/r_{DZ})
systolischer BD Ruhe, mmHg	114.5 ± 17.3	112.1 ± 13.7	0.24 (0.55/0.43)
diastolischer BD Ruhe, mmHg	63.1 ± 11.1	61.8 ± 8.5	0.40 (0.65/0.45)
Herzfrequenz Ruhe, bpm	71.9 ± 11.0	71.7 ± 9.5	0.54 (0.53/0.35)
Systolischer BD Rechnen, mmHg	134.8 ± 20.1	132.9 ± 19.5	0.34 (0.59/0.42)
Diastolischer BD Rechnen, mmHg	75.4 ± 11.9	74.5 ± 9.6	0.48 (0.65/0.41)
Herzfrequenz Rechnen, bpm	88.8 ± 16.6	88.8 ± 15.4	0.42 (0.52/0.31)
Systolischer BD Cold Pressor, mmHg	128.9 ± 19.2	123.6 ± 16.9	0.30 (0.55/0.40)
Diastolischer BD Cold Pressor, mmHg	74.7 ± 11.8	70.9 ± 10.2	0.59 (0.59/0.31)
Herzfrequenz Cold Pressor, bpm	77.1 ± 11.9	76.8 ± 10.5	0.40 (0.60/0.40)
SysBD Anstieg Rechnen, mmHg	15.2 ± 16.1	13.7 ± 14.6	0.00(0.17/0.23)
DiaBD Anstieg Rechnen, mmHg	12.3 ± 7.4	12.6 ± 7.7	0.12 (0.25/0.19)
HF Anstieg Rechnen, bpm	17.0 ± 11.2	17.2 ± 10.1	0.52 (0.52/0.26)
SysBD Anstieg Cold Pressor, mmHg	11.3 ± 11.9	8.8 ± 9.6	0.08 (0.29/0.25)
DiaBD Anstieg Cold Pressor, mmHg	11.7 ± 7.2	9.4 ± 6.1	0.30 (0.45/0.30)
HF Anstieg Cold Pressor, bpm	5.2 ± 5.2	5.1 ± 4.1	0.40 (0.46/0.26)

Nach dem Nachweis genetischer Einflüsse auf das Blutdruckniveau sowie den Blutdruckanstieg wurde die Frage untersucht, ob die zugrundeliegenden Faktoren jeweils spezifisch für Niveau und Anstiegsregulation sind, oder ob sich eine Gruppe von Genen nachweisen läßt, die beide Komponenten der Blutdruckregulation beeinflußt. Die für den Cold-Pressor Test berechnete bivariate Heritabilitätsanalyse (Tabelle 6) ergab keine signifikante genetische Korrelation zwischen Blutdruckniveau und – anstieg, es muss davon ausgegangen werden, dass unterschiedliche Gene in den verschiedenen Regulationssystemen involviert

sind. Dieses Ergebnis hat große Bedeutung für die Auswahl von Kandidatengenen in nachfolgenden molekulargenetischen Studien.

Tabelle 6: Gemeinsame und spezifische genetische Einflüsse für Ruheblutdruck und Blutdruckanstieg während des Cold-Pressor Tests (CP).

	gemeinsame genetische Einflüsse	spezifische genetische Einflüsse	gemeinsame Umweltfaktoren	spezifische Umweltfaktoren
sys BD Ruhe	0.52	-	0.48	-
∆sys BD CP	0	0.55	0.02	0.42
dia BD Ruhe	0.51	-	0.48	-
∆dia BD CP	0	0.52	0.01	0.46

Nach dem Nachweis genetischer Einflüsse auf Ruheblutdruck und situationsbedingte Blutdruckvariation wurde mit der Baroreflex-Sensitivität ein weiterer Aspekt der Blutdruckregulation untersucht (28). Auch für diesen Phänotyp gab es erwartungsgemäß keine signifikanten Unterschiede zwischen MZ und DZ (Tabelle 7).

Tabelle 7: Ergebnisse der Berechnung der Baroreflex-Sensitivität (Mittelwert ± SD).

Parameter	MZ	DZ
BRSLF	17 ± 13	16 ± 9
BRSHF	21 ± 18	20 ± 14
BRS+	19 ± 16	18 ± 10
BRS-	21 ± 15	20 ± 11

BRSLF: Mittelwert der Transferfunktion im Kreuzspektrum im unteren Frequenzbereich (0.04-0.15 Hz), BRSHF: Mittelwert der Transferfunktion im oberen Frequenzbereich (0.15-0.4 Hz), BRS+: mittlerer Regressionskoeffizient bei Anstieg, BRS-: mittlerer Regressionskoeffizient bei Abfall.

Die Baroreflexfunktion zeigte in der untersuchten Stichprobe eine signifikante Beziehung zu Bodymass-Index, Blutdruck und physischer Aktivität gemessen über die Stunden pro Woche (Tabelle 8), die Berechnung der Heritabilität der Baroreflex-Sensitivität erfolgte daher zusätzlich nach Korrektur für diese Faktoren (Tabelle 9).

Tabelle 8: Kreuzkorrelation zwischen Baroreflex-Sensitivität (am Beispiel von BRSLF), BMI, Blutdruck, und physischer Aktivität mit zugehörigem Signifikanzniveau (p).

	r (Pearson)	p
BRSLF vs. BMI	- 0.193	<0.001
BRSLF vs. sysBD	- 0.335	<0.001
BRSLF vs. diaBD	- 0.319	<0.001
BRSLF vs. phys.Aktivität.	0.166	<0.02

Tabelle 9: Korrelationskoeffizienten (r) und Heritabilität (h^2) der Baroreflex-Sensitivität bei MZ und DZ vor und nach Korrektur für BMI und Ruheblutdruck (c).

	r MZ	r DZ	h^2	r MZc	r DZc	h^2c
BRSLF	0.48	0.05	0.43*	0.43	0.13	0.41*
BRSHF	0.44	- 0.03	0.40*	0.37	0.08	0.35*
BRS+	0.46	0.03	0.42*	0.40	0.16	0.39*
BRS-	0.38	0.09	0.36*	0.29	0.20	0.18

* $p<0.05$

Die Korrektur der Baroreflexmaße für BMI und Ruheblutdruck führte lediglich bei einem der verwendeten Indizes (BRS-) zu einer signifikanten Reduzierung der Heritabilität, so dass spezifische genetische Faktoren für den Baroreflex anzunehmen sind.

Als weiteres Maß der Kreislaufregulation wurde die Herzfrequenzvariabilität berechnet (29). Während die Herzfrequenz selbst nur in geringem Maß durch genetische Faktoren beeinflusst wird, lassen sich für die rhythmischen Veränderungen genetische Einflüsse auf die zugrunde liegenden physiologischen Systeme nachweisen (Tabelle 10).

Tabelle 10: Ergebnisse der Heritabilitätsberechnung für die Herzfrequenzvariabilität (MZ=95, DZ=46 Paare), mit additiven (a^2) und nichtadditiven (d^2) genetischen Einflüssen sowie gemeinsamen (c^2) und getrennten (e^2) Umwelteinflüssen.

	a^2	d^2	c^2	e^2	r_{mz} / r_{DZ}
Schlagintervall	0.17	-	0.37	0.46	0.58 / 0.38
SDNN	-	0.60	-	0.40	0.64 / 0.22
RMSSD	-	0.65	-	0.35	0.68 / 0.13
Total Power	0.56		-	0.44	0.60 / 0.24
ULF+VLF+LF	-	0.47	-	0.53	0.50 / 0.05
HF	0.39		-	0.61	0.40 / 0.21
PNNL10	-	0.56	-	0.44	0.63 / 0.06
PNNL20	-	0.59	-	0.41	0.63 / 0.06
PNN100	-	0.58	-	0.42	0.58 / 0.16-

Schlagintervall: Mittelwert über 30 Min; SDNN: Standardabweichung der Schlagintervalle; RMSSD: Wurzel aus dem mittleren Quadrat der Differenz zweier aufeinanderfolgender Herzschläge; Gesamtpower: Power über das gesamte Frequenzspektrum; ULF, VLF, und LF: Power im Frequenzbereich von 0-0.0033 Hz, 0.0044-0.04, und 0.04-0.15 Hz; HF: normalisierte Power im Frequenzbereich von 0.15 bis 0.4 Hz; PNNL10, 20 und 100: Prozentanteil von Herzschlägen mit einer Differenz von weniger als 10 und 20 ms bzw. mehr als 100 ms.

Ein weiterer Bereich kardiovaskulärer Phänotypen, der im Rahmen der Zwillingsstudie untersucht wurde, betrifft die Elektrophysiologie des Herzens (30). Insbesondere die Reizleitungszeit QT bzw. QTc nach Korrektur für die Herzfrequenz stand im Mittelpunkt des Interesses, da eine pathophysiologische Relevanz für Herzrhythmusstörungen und plötzlichen Herztod diskutiert wird. Weiterhin gibt es monogene Formen einer pathologisch verlängerten Reizleitung, Long-QT Syndrom, hier sind eine Reihe von beteiligten Genen bekannt, für die die genetische Varianz in der Bevölkerung unbekannt ist. Der Vergleich zwischen MZ und DZ ist in Tabelle 11 dargestellt. Die Ergebnisse der Heritabilitätsberechnung folgt in Tabelle 12. Für die Reizleitungszeit beträgt der Anteil der genetischen Varianz etwa 50%, während für die Herzfrequenz (hier das RR-Intervall) keine genetischen Einflüsse nachweisbar waren.

Tabelle 11: EKG-Parameter bei MZ und DZ.

Variable	MZ (Mittelwert ± SD)	DZ (Mittelwert ± SD)
n (Paare)	125	75
P (msec)	110 ± 11	106 ±12*
PR (msec)	152 ± 20	151 ±22
QRS (msec)	99 ± 10	97 ± 11
QTc (msec)	414 ± 25	416 ± 25
P Axis (degrees)	44 ± 20	43 ± 21
QRS Axis (degrees)	55 ± 25	49 ± 26
T Axis (degrees)	37 ± 18	33 ± 16

* $p<0.01$

Tabelle 12: Angaben zu Heritabilität (a^2 und zugehörige Irrtumswahrscheinlichkeit p) und Umwelteinflüssen (gemeinsame Umweltfaktoren c^2, getrennte Umweltfaktoren e^2) sowie zugrundeliegende Korrelationen bei MZ und DZ für Komponenten des EKG.

	a^2	p	c^2	e^2	r_{MZ}	r_{DZ}
RR		n.s.	0.46	0.54	0.50	0.37
P	0.46	0.01	0.12	0.42	0.55	0.40
PR		n.s.	0.53	0.47	0.47	0.51
QRS	0.40	0.01		0.60	0.41	0.08
QTc	0.52	0.01		0.48	0.52	0.30
P Axis		n.s.	0.23	0.77	0.26	0.29
QRS Axis	0.59	0.01		0.41	0.60	0.24
T Axis	0.52	0.01		0.48	0.51	0.11

3.1.2 Lipide

Da hohe Konzentrationen von Gesamtcholesterin, LDL-Cholesterin und Triglyzeriden sowie niedrige HDL-Cholesterinspiegel als Risikofaktoren nachgewiesen wurden (z.B. in der PROCAM-Studie, 31), wurde in die Untersuchung genetischer Einflüsse auf das Risiko für Herz-Kreislauferkrankungen auch die Blutfette einbezogen (32,33).

Es traten keine signifikanten Unterschiede zwischen MZ und DZ in den untersuchten Lipidwerten auf (Tabelle 13).

Tabelle 13: Serum-Lipide bei MZ und DZ.

Variable	MZ (Mittelwert ± SD)	DZ (Mittelwert ± SD)
n (Paare)	122	100
Gesamtcholesterin mg/dl)	183 ± 39	193 ± 42
HDL-Cholesterin (mg/dl)	51 ± 14	57 ± 17
LDL-Cholesterin (mg/dl)	115 ± 34	115 ± 32
Triglyzeride (mg/dl)	87 ± 67	100 ± 64

Cholesterin: mmol/L=0.01129*mg/dl, Triglyzeride: mmol/L=0.0286*mg/dl

Für alle untersuchten Lipidwerte konnte ein signifikanter genetischer Einfluss nachgewiesen werden, die Heritabilität lag zwischen 59% und 72% (Tabelle 14). Dieser Nachweis starker genetischer Einflüsse bildete die Grundlage nachfolgender Untersuchungen von Kandidatengenen.

Tabelle 14: Genetische und Umwelteinflüsse auf die Lipidwerte sowie zugrundeliegende Korrelationen bei MZ und DZ

	genetische Einflüsse a^2	p	Umwelt-einflüsse e^2	r_{MZ}	r_{DZ}
Gesamt-Cholesterol	0.64	0.01	0.36	0.65	0.37
HDL- Cholesterol	0.59	0.01	0.41	0.63	0.30
LDL –Cholesterol	0.66	0.01	0.34	0.66	0.36
Triglyceride	0.72	0.01	0.28	0.72	0.44

3.1.3 Psychologische Variablen

Persönlichkeitsmerkmale können die Gesundheit auf verschiedenste Weise beeinflussen. Ein Bereich mit besonderer Bedeutung ist die Stressbewältigung (Coping), da einerseits äußere Belastungen als Krankheitsrisiko durch die mentale Bewertung/Verarbeitung gepuffert oder verstärkt werden können, andererseits Krankheiten selbst als Stress wirksam werden und ihre Bewältigung den Verlauf beeinflusst (34). Coping als Persönlichkeitsbereich weist sowohl stabile Grundtendenzen als auch situations- und entwicklungsbedingte Veränderungen auf. In der medizinischen und psychologischen Fachliteratur wurde die Stressverarbeitung implizit oder explizit als gelerntes Verhalten betrachtet, das primär durch familiäre

Umweltfaktoren beeinflusst wird (35). Diese Annahme erschien wenig gerechtfertigt, da für viele Persönlichkeitsmerkmale ein substantieller Anteil genetischer Einflüsse nachweisbar ist und die Stressverarbeitung in Beziehung zu anderen Persönlichkeitsmerkmalen steht (36). In einer Studie an weiblichen Zwillingen wurde für zwei von drei Copingskalen eine Heredität von 30% berechnet (37). In einer Studie an Kindern zeigten 4 von 7 Copingskalen genetische Einflüsse (38).

Zur Untersuchung der Frage genetischer Einflüsse auf die Stressbewältigung untersuchten wir 19 Copingstrategien. Zur Verringerung statistischer Probleme durch Mehrfachtestung wurden aus den 19 Skalen mittels Faktoranalyse 4 Sekundärskalen berechnet. Signifikanztests und Tests auf genetische Korrelation wurden für die Sekundärfaktoren durchgeführt, die Hereditätsberechnung der 19 Einzelskalen dient der Ableitung von Hypothesen über differenzielle Einflüsse von Genen und Umweltfaktoren (Tabelle 15).

Alle Sekundärfaktoren zeigten einen signifikanten Einfluß genetischer Faktoren (20% bis 50% der Varianz), gemeinsame Umweltfaktoren waren nicht nachweisbar. Von den 19 Einzelskalen zeigten 3 Skalen Einflüsse von genetischen und gemeinsamen Umweltfaktoren, 14 Skalen zeigten nur genetische Einflüsse und 2 Skalen zeigten nur Einflüsse gemeinsamer Umweltfaktoren (39).

Tabelle 15: Ergebnisse der Heritabilitätsberechnung der Copingskalen des SVF.

SVF Skala	a^2	d^2	c^2	e^2	rMZ/rDZ	χ^2 / df
Faktor1 Verteidigung		0.52		0.48	0.47 / 0.25	6.8 / 4
Faktor2 Emotionales Coping		0.23		0.77	0.29 / 0.04	4.4 / 4
Faktor3 Ersatz		0.41		0.59	0.42 / -0.09	3.9 / 4
Faktor4 Aktives Coping	0.21		0.09	0.70	0.32 / 0.17	2.3 / 3
Bagatellisierung		0.23		0.77	0.25 / 0.03	3.3 / 4
Herunterspielen durch Vergleich		0.43		0.57	0.45 /-002	1.2 / 4
Schuldabwehr		0.33		0.67	0.35 /-0.05	4.7 / 4
Ablenkung von Situationen			0.14	0.86	0.13 / 0.17	0.5 / 4
Ersatzbefriedigung	0.21			0.79	0.19 / 0.13	2.4 / 4
Suche nach Selbstbestätigung		0.28		0.72	0.28 / 0.00	1.4 / 4
Situationskontrollversuche	0.39			0.61	0.41 / 0.20	4.7 / 4
Reaktionskontrollversuche		0.21		0.79	0.23 /-0.05	3.5 / 4
Positive Selbstinstruktion		0.35		0.65	0.36 / 0.11	3.1 / 4
Bedürfnis nach sozialer Unterstützung		0.36		0.64	0.36 / 0.14	5.9 / 4
Vermeidungstendenz			0.29	0.71	0.29 / 0.29	3.0 / 4
Fluchttendenz		0.50		0.50	0.50 / 0.15	0.2 / 4
Soziale Abkapselung		0.37		0.63	0.35 / 0.13	1.4 / 4
Gedankliche Weiterbeschäftigung		0.45		0.55	0.43 / 0.22	3.5 / 4
Resignation		0.53		0.47	0.52 / 0.22	2.1 / 4
Selbstbemitleidung	0.21		0.15	0.64	0.35 / 0.27	2.6 / 3
Selbstbeschuldigung		0.35		0.65	0.36 /-0.04	6.3 / 4
Aggression			0.39	0.61	0.37 / 0.41	3.9 / 4
Pharmakaeinnahme	0.45			0.55	0.45 / 0.22	1.0 / 4

Der Test auf gemeinsame genetische Faktoren erfolgte mit Hilfe einer Varianzaufteilung nach Cholesky. Nach Modellierung eines vollständigen Modells mit genetischen und Umweltfaktoren sowohl spezifisch als auch gemeinsam für die 4 Sekundärskalen des SVF (Modell I) wurden in einem ersten Analyseschritt sämtliche Einflüsse der von Geschwistern gemeinsam erlebten Umweltfaktoren aus dem Modell entfernt (Modell II, Tabelle 16). Da die Güte der Modellanpassung nicht signifikant reduziert wurde (Tabelle 17), erfolgt der Vergleich der folgenden Modelle mit Modell II. Im Modell III wurden die genetischen Kovarianzen entfernt, getes-

tet wurde somit das Vorhandensein genetischer Faktoren, die mehrere Copingskalen beeinflussen. Dieses Modell zeigte eine signifikante Verschlechterung der Anpassung an die empirischen Daten, ebenso wie Modell IV. In diesem Fall wurde nur ein gemeinsamer genetischer Faktor für die 4 Copingskalen modelliert ohne spezifische genetische Einflüsse auf die einzelnen Bewältigungsstrategien.

Tabelle 16: Varianz-Kovarianz Matrix für genetische und Umweltfaktoren für das Modell II (keine gemeinsamen Umweltfaktoren), die genetische Varianz (Heritabilität) ist in der oberen Diagonalen dargestellt, die Kovarianzen werden unterhalb der Diagonalen angegeben.

Genetische Faktoren	SVF1	SVF2	SVF3	SVF4
1	0.36			
2	-0.10	0.48		
3	-0.05	0.17	0.32	
4	0.16	-0.10	-0.07	0.29
Umweltfaktoren				
1	0.64			
2	-0.01	0.52		
3	0.23	0.21	0.68	
4	0.22	0.19	0.24	0.71

Tabelle 17: Parameter der Modellanpassung der multivariaten Analyse (* vs. Modell I, ** vs. Modell II)

Modell	χ^2 / df	p	change in χ^2 / df	p
I. Vollständiges Modell ACE	49.1 / 42	0.21		
II. keine gemeinsamen Umweltfaktoren	49.8 / 52	0.56	0.7 / 10	n.s.*
III. nur spezifische genetische Faktoren	71.9 / 58	0.10	22.1 / 6	0.01**
IV. nur 1 gemeinsamer genetischer Faktor	125.7 / 58	0.00	75.9 / 6	0.01**

Die Analyse ergab, dass die verschiedenen Bewältigungsstrategien sowohl durch gemeinsame genetische Faktoren beeinflusst werden als auch jeweils spezifische genetische Einflüsse aufweisen. Weitere differenzierte Analysen setzen einen größeren Stichprobenumfang voraus. Insbesondere die gemeinsame Analyse von

Coping und weiteren Persönlichkeitsmerkmalen wird weitere Aufschlüsse über die genetische Struktur der Stressbewältigung erbringen und somit die Möglichkeiten der funktionellen Untersuchung einzelner Gene verbessern.

3.2 Kopplungs- und Assoziationsanalysen

3.2.1 Kardiovaskuläre Parameter

Verschiedene Genloci mit Kandidatengenen für die Blutdruckregulation bzw. Hypertonie wurden untersucht. Aus dem Renin-Angiotensin-System wurde das Renin-Gen ausgewählt, das im Tierversuch als Blutdruck-QTL nachgewiesen wurde (40), das Angiotensinogen-Gen, für das ein Einfluss auf die Hypertonie diskutiert wird (41,42), sowie die Gene für das Angiotensin-Konversions-Enzym ACE und den AT_1-Rezeptor, die mit erhöhtem kardiovaskulärem Risiko assoziiert sind (43).

Weiterhin wurden Kandidatengene aus monogenen Formen der Hypertonie ausgewählt. Zum einen wurde das Liddle-Syndrom ausgewählt (44), das durch die Hyperaktivität des epithelialen amyloride-sensitiven Natriumkanals im distalen renalen Tubulus verursacht wird durch Salz- und Volumenretention. Zum anderen untersuchten wir die Genregion auf Chromosom 12, in die die autosomal-dominante Hypertonie mit Brachydaktylie kartiert wurde (45), das Gen selbst ist bisher unbekannt.

Der Genort für Lipoprotein-Lipase ist ein nachgewiesener genetischer Risikofaktor für Hypertriglyceridämie (46) und möglicherweise relevant für die familiäre dyslipidämische Hypertonie (47).

IGF-1 (Somatomedin C) wurde in erhöhter Konzentration im Blutplasma von Patienten mit essentieller Hypertonie nachgewiesen und ist korreliert mit dem Natrium/Kalium Countertransport (48).

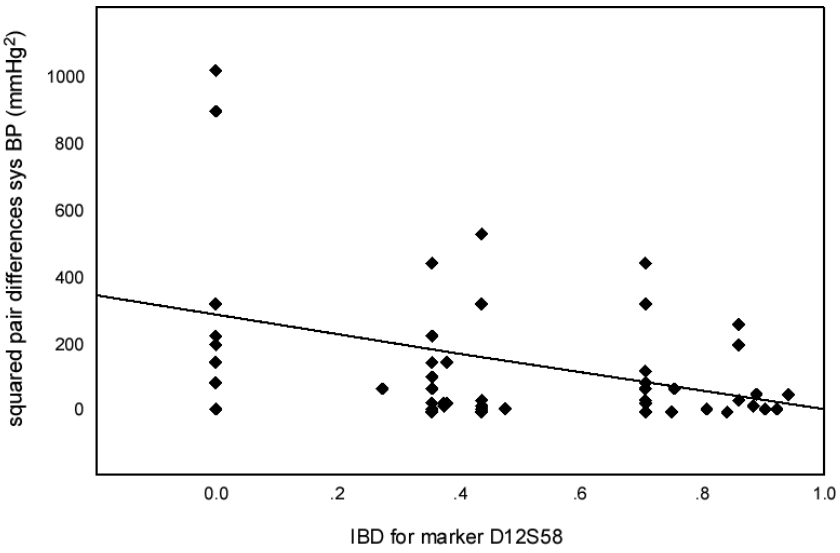
Adducin ist ein α/β heterodimeres Cytoskeletonprotein, das in verschiedenen Geweben vorkommt, unter anderem in der Niere. Das Protein ist an der Regulation der Signaltransduktion der Zelle beteiligt. Veränderungen im Adducin beeinflussen die Oberflächenexpression und Geschwindigkeit der Natrium-Kalium-Pumpe. Punktmutationen in den α– und β-Adducin Untereinheiten erklären bis zu 50% des Blutdruckunterschiedes zwischen den Milan Hypertonie- und Normotonie- Ratten-

stämmen (49). Sowohl Assoziationsstudien (50) als auch Kopplungsstudien (7) bringen Adducin mit Hypertonie in Verbindung.

Der β–2 adrenerge Rezeptor (β–2 AR) wird im Zusammenhang mit essentieller Hypertonie diskutiert, sowohl auf der Grundlage von Studien zur β–2 AR beeinflussten Vasodilatation (51) als auch nach molekulargenetischen Assoziationsstudien (52,53).

Details zu den analysierten Mikrosatelliten-Markern und Polymorphismen sind in den jeweiligen Publikationen (24,25,26) angegeben. Ein Beispiel für die Kopplungsanalyse nach Haseman-Elston ist in Abbildung 6 dargestellt für einen Mikrosatelliten im IGF1-Lokus.

Abbildung 6: Beispiel einer Haseman-Elston Regression. Die Ordinate zeigt die quadrierte Paardifferenz des systolischen Blutdrucks, die Abszisse gibt die genetische Übereinstimmung („*Pi-hat*" als normierte IBD) an für einen Marker im IGF1-Lokus.



Die Ergebnisse der verschiedenen Studien werden tabellarisch zusammengefasst (Tabelle 18). Für den systolischen Blutdruck konnten die Genloci von IGF-1, Liddle Syndrom, AT_1 Rezeptor als QTL nachgewiesen werden, für den diastolischen Blutdruck der Genort für autosomal-dominante Hypertonie mit Brachydactyly. Sowohl systolischer als auch diastolischer Blutdruck sind gekoppelt mit den Genorten für Renin, Adducin und β-2 AR.

Tabelle 18: Ergebnisse der Kopplungsanalyse zwischen dem Blutdruck (korrigiert für Geschlecht und Alter) und Kandidatengenen. Es wird jeweils die Irrtumswahrscheinlichkeit p angegeben.

Genlocus	sys BD sitzend	sys BD stehend	sys BD liegend	dia BD sitzend	dia BD stehend	dia BD liegend
IGF1 Chromosom 12	0.00005	0.0002	0.004	n.s.	n.s.	n.s.
Liddle Chromosom 16	0.026	0.010	n.s.	n.s.	n.s.	n.s.
AT_1 Rezeptor Chromosom 3	0.0008	n.s.	0.0026	n.s.	n.s.	n.s.
LPL Chromosom 8	n.s.	n.s.	0.005	n.s.	n.s.	0.061
ACE Chromosom 17	n.s.	n.s.	0.051	n.s.	n.s.	n.s.
Hypertonie/ Brachydactylie Chromosom 12	n.s.	n.s.	n.s.	0.0012	0.024	0.0024
Renin Chromosom 1	0.0009	0.000001	0.0018	0.0036	0.009	0.008
AGT Chromosom 1	n.s.	n.s.	n.s.	0.038	n.s.	n.s.
Adducin Chromosom 4	0.008	0.001	0.016	n.s.	n.s.	n.s.
ß-2 AR Chromosom 5	n.s.	0.001	0.001	0.001	0.001	n.s.

Sowohl für das α-Adducin-Gen als auch das Gen für den β-2 AR wurden zusätzlich zur Kopplungsanalyse auch Assoziationsanalysen durchgeführt. Im Adducin-Gen wurde der 460Gly/Trp Polymorphismus untersucht, es ergab sich keine signifikante Beziehung zum Blutdruck. Für den β-2 AR wurden folgende Polymorphismen genotypisiert: Arg16/Gly, Gln27/Glu, Thr164/Ile, und eine Variante in der Promotorregion (-47C/T). Alle Polymorphismen zeigten eine Beziehung zum Blutdruck. Alle Polymorphismen sind im Kopplungsungleichgewicht zueinander. Konditionale Analysen verweisen auf den Arg16/Gly Polymorphismus als wahrscheinlich funktionell bedeutendste Variation.

Neben dem Blutdruck wurde auch die Herzgröße als Phänotyp im Zusammenhang mit dem IGF1-Gen, dem Angiotensinogen-Gen und dem ACE-Gen analysiert. Es ergab sich eine signifikante Kopplung für IGF1 und eine Assoziation für den I/D-Polymorphismus im ACE-Gen.

Für Parameter der Reizleitung im Herzen (QT-Zeit) wurden als Kandidatengene für Kopplungsanalysen 5 Loci gewählt, die für monogene Formen des Long-QT Syndroms bedeutsam sind (54). Für die Loci LQT1 und LQT4 konnte ein Zusammenhang mit der frequenzkorrigierten Reizleitungszeit QTc nachgewiesen werden, LQT2 und LQT3 zeigten eine Beziehung zur elektrischen Herzachse (Tabelle 19) (30).

Tabelle 19: Ergebnisse der Kopplungsanalysen für die EKG-Parameter.

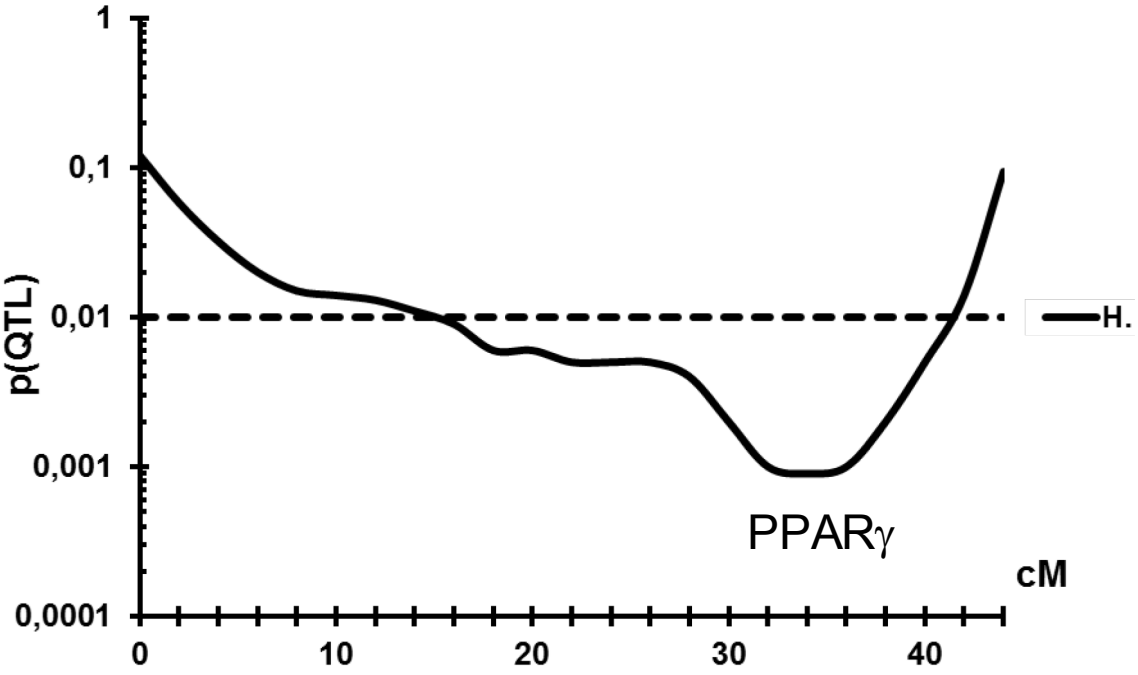
Lokus	QTc	P-Welle	QRS	QRS Achse	T Achse
LQT1	< 0.001	0.99	0.06	0.99	0.99
LQT2	0.76	0.99	0.99	< 0.001	0.004
LQT3	0.17	0.99	0.43	0.003	0.99
LQT4	< 0.001	0.99	0.25	0.05	0.42
LQT5	0.99	0.17	0.06	0.99	0.55

Diese Ergebnisse verweisen auf die mögliche Bedeutung dieser Gene über die seltenen monogenen Krankheitsformen hinaus für Herzrhythmusstörungen und plötzlichen Herztod.

3.2.2 Lipide

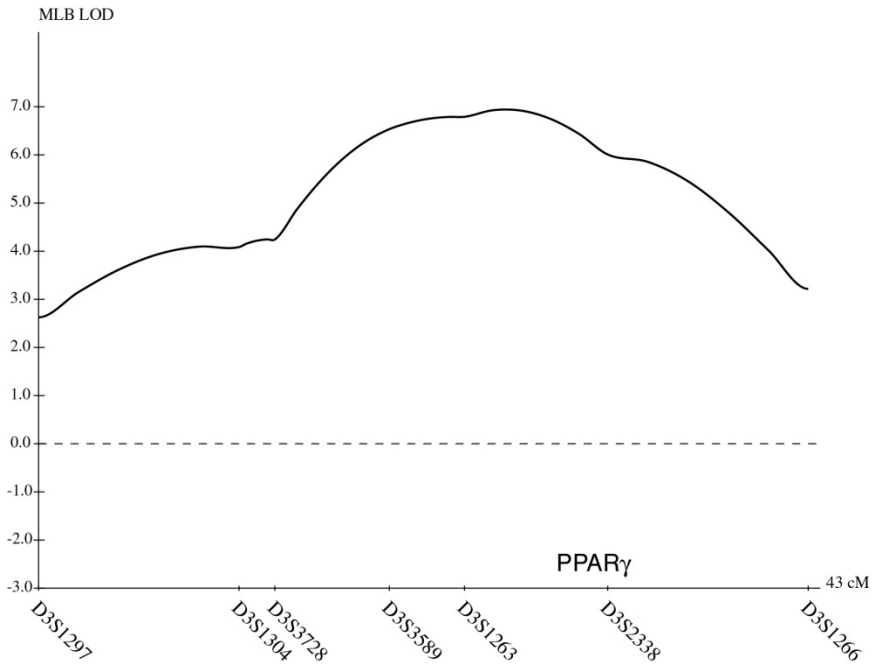
Als Kandidatengene für Lipidspiegel wurden die Gene des Makrophagen-Scavenger-Receptors (MSR), des LDL-Rezeptors (LDL-R), der Lipoprotein-Lipase (LPL) sowie des Peroxisome-Proliferator-Activated Rezeptors (PPARγ) untersucht (32,33). Durch Kopplungsanalysen konnte gezeigt werden, dass MSR die Konzentration von HDL-Cholesterin beeinflusst ($p<0.001$). PPARγ zeigte Kopplung mit LDL-Cholesterin ($p<.005$), HDL-Cholesterin ($p<.0009$, Abbildung 7) und BMI ($p<0.005$), sowie Assoziation zu HDL-Cholesterin ($p<0.001$) und BMI ($p<0.03$).

Abbildung 7: Beispiel für die Kopplungsanalyse mittels Varianz-Kovarianzanalyse. Die Ordinate gibt die Irrtumswahrscheinlichkeit für einen Zusammenhang (Kopplung) an, die Abszisse beschreibt den untersuchten Chromosomenabschnitt.



Bei der Analyse der Kopplungsdaten für PPARγ beobachteten wir eine Abweichung zwischen erwarteter und empirischer genetischer Übereinstimmung (IBD) bei den dizygoten Zwillingen. Zur weiteren Analyse dieser Beobachtung wurden Zwillingspaare aus Finnland und Polen sowie Geschwisterpaare analysiert. Der Status ‚dizygoter Zwilling' wurde als qualitativer Phänotyp definiert. In einer *affected sib pair* Kopplungsanalyse konnte ein Zusammenhang zwischen diesem Status und der PPARγ-Region auf Chromosom 3 nachgewiesen werden (LOD=6.93, Abbildung 8) (55).

Abbildung 8: LOD-Kurve der Kopplungsanalyse mit dem Phänotyp ‚dizygoter Zwilling' und dem PPARγ-Lokus



Aufgrund der physiologischen Funktionen von PPARγ in der Regulation des Fettstoffwechsels lässt sich die Hypothese aufstellen, dass übereinstimmende Genotypen bei Mehrlingsschwangerschaften die Überlebenswahrscheinlichkeit beider Föten erhöht, die Wahrscheinlichkeit sogenannter ‚*vanishing twins*' also geringer ist. Unterschiede in der intrauterinen Entwicklungsgeschwindigkeit könnten zu einer präferenziellen Versorgung des weiterentwickelten Fötus führen.

3.2.3 Psychologische Variablen

Ausgehend von Ergebnissen zur genetischen Beeinflussung der Stressverarbeitung testeten wir die Hypothese, dass das Gen des β-2 adrenergen Rezeptors (β-2 AR) Copingstile beeinflusst (56). Wir testeten Kopplung mit Hilfe von Mikrosatelliten-Markern sowie Assoziation mit allen Single Nucleotide Polymorphismen (SNPs) im Gen, die in einem Aminsäureaustausch resultieren. Wir konnten Kopplung nachweisen für den Sekundärfaktor Ersatzbefriedigung, signifikante Assoziationen traten auf zwischen dem -47C/T SNP in der regulatorischen Region des β2-AR Gens und mehreren Copingstilen (Tabelle 20).

Tabelle 20: Beziehung zwischen β2-AR Gen und Coping

Skala	ct1541	ag1633	cg1666	ga2078
Faktor1 Verteidigung	<0.05		<0.05	
Faktor2 Emotionales Coping	<0.05		<0.10	
Faktor3 Ersatz	<0.01		<0.05	
Faktor4 Aktives Coping	<0.10			<0.10
Bagatellisierung	<0.05			<0.05
Herunterspielen durch Vergleich	<0.05	<0.01	<0.10	
Schuldabwehr	<0.01		<0.05	
Ablenkung von Situationen	<0.10		<0.10	
Ersatzbefriedigung				
Suche nach Selbstbestätigung	<0.05		<0.10	
Situationskontrollversuche				
Reaktionskontrollversuche	<0.10			<0.10
Positive Selbstinstruktion	<0.01		<0.05	<0.05
Bedürfnis nach sozialer Unterstützung				
Vermeidungstendenz				
Fluchttendenz	<0.05		<0.05	
Soziale Abkapselung		<0.05		<0.01
Gedankliche Weiterbeschäftigung	<0.05	<0.05	<0.05	
Resignation	<0.05		<0.05	
Selbstbemitleidung				
Selbstbeschuldigung				
Aggression				
Pharmakaeinnahme	<0.10		<0.10	<0.10

Da zwischen Coping und dem Blutdruck eine Korrelation besteht, verweisen die Ergebnisse auf mögliche biologische Grundlagen der Beziehung zwischen psychischen und physiologischen Risikofaktoren für Herz-Kreislauf-Erkrankungen.

4 Diskussion und Ausblick

Ausgangspunkt der hier dargestellten Studien war die Hypothese, dass genetische Einflüsse nicht nur bei der Krankheitsentstehung eine Rolle spielen, sondern auch bei krankheitsrelevanten physiologischen Kennwerten einen nachweisbaren Einfluss haben. Diese Hypothese beruht auf zahlreichen vorangegangenen Zwillings- und Familienuntersuchungen und konnte durch eigene Daten bestätigt werden. Von besonderer Bedeutung sind die Analysen von Phänotypen, bei denen es keine oder nur unzureichende Vorstudien gab, z.B. für die Baroreflex-Sensitivität, die Reizleitungszeit QT oder auch die psychologische Stressbewältigung.

Diese ‚konventionellen' Zwillingsanalysen wurden in den vorgelegten Studien erweitert durch molekulargenetische Analysen, deren Methoden aus Geschwisterpaaranalysen abgeleitet wurden. Sowohl für Kopplungs- als auch Assoziationsanalysen wurden nicht Diagnosen als qualitative Merkmale zugrunde gelegt, sondern quantitativ bestimmte physiologische Parameter. Der Übergang von qualitativen zu quantitativen Phänotypen ist notwendig, da nur diese Betrachtungsweise der Biologie komplexer Erkrankungen gerecht wird, und er vergrößert gleichzeitig die Erfolgsaussichten der Analysen durch den Informationsgewinn. Dieser methodische Weg wird als QTL-Analyse - neuerdings im Kontext von Assoziationsstudien auch QTN-Analyse (*quantitative trait nucleotide)* – bezeichnet. Methodische Grundlagen wurden sowohl im Rahmen von Pflanzen- und Tiergenetik als auch im Rahmen der humanen Verhaltensgenetik erarbeitet (57) und finden ihren Weg erfolgreich auch im Rahmen der medizinischen Humangenetik.

Die in den dieser Arbeit zugrundeliegenden Publikationen dargestellten Ergebnisse belegen nicht nur die praktische Realisierbarkeit von Kopplungs- und Assoziationsanalysen an unausgelesenen gesunden Probanden, sie belegen auch an verschiedenen Beispielen, dass monogene Erkrankungen und polygene komplexe Erkrankungen nicht notwendigerweise grundsätzlich verschieden sind. So konnte für das Liddle-Syndrom und das Long-QT Syndrom nachgewiesen werden, dass beteiligte Gene auch als QTLs in der Normalbevölkerung wirksam werden. Krankheitsrelevante Mutationen stellen hier seltene Polymorphismen mit starker physiologischer Auswirkung dar, weitere Polymorphismen dieser Gene haben ebenfalls physiologischen Einfluss, allerdings in einem geringeren Ausmaß. Dieser Nachweis belegt den Wert unserer Forschungsstrategie der QTL-Kartierung an Gesunden mit anschließender Suche nach pathophysiologisch relevanten Polymorphis-

men. In der Mehrzahl der komplexen Erkrankungen wird hier vor allem die Kombination von funktionell relevanten Polymorphismen in verschiedenen Genen eines Regelsystems zur Zielgröße. Dabei sind sowohl additive Genwirkungen als auch Epistasiseffekte zu erwarten. Insoweit sind die vorgelegten Arbeiten nur ein erster Schritt hin zu multivariaten Analysen, die verschiedenste Kenngrößen physiologischer Systeme und mehrere beteiligte Gene simultan einbeziehen. Auch auf der Ebene der Gene selbst muss die Komplexität erhöht werden, nicht die Analyse einzelner Polymorphismen, sondern nur die Einbeziehung ihrer Beziehungen in Haplotypen wird der biologischen Wirklichkeit gerecht.

Solche multivariaten Analysen verlangen Stichprobengrößen von 500 bis zu mehreren tausend Geschwisterpaaren. Der Ausbau unseres Untersuchungskollektivs ist konsequenterweise ein Schwerpunkt der gegenwärtigen Arbeit. Parallel dazu müssen aber auch die analytischen Werkzeuge verbessert werden. Neben der zunehmenden Automatisierung der Laborarbeit setzen große Stichproben auch eine Automatisierung der Datenaufbereitung und Auswertung voraus. International ist dies gegenwärtig der Flaschenhals großer genetischer Studien.

Die hier vorgelegten Arbeiten beweisen, dass solche Schwierigkeiten überwindbar sind, wenn eine enge Kooperation zwischen Klinik, Molekulargenetik und genetischer Epidemiologie mit gegenseitigem Verständnis erreicht werden kann. Der Weiterführung der Studien wird daher mit großer Erwartung entgegengesehen.

Literaturverzeichnis

1: Ball D, Hill L, Freeman B, Eley TC, Strelau J, Riemann R, Spinath FM, Angleitner A, Plomin R: The serotonin transporter gene and peer rated neuroticism. Neuroreport. 8, 1997, 1301-1304.

2: Becker A, Busjahn A, Faulhaber HD, Bähring S, Robertson J, Schuster H, Luft FC: Twin zygosity: Automated determination with microsatellites. J Reprod Med. 42, 1997, 260-266.

3: Jahnke W, Erdmann G, Kallus W: Der Streßverarbeitungs-Fragebogen. Göttingen, Verlag für Psychologie Dr. C.J. Hogrefe, 1985.

4: Rigat B, Hubert C, Alhenc Gelas F, Cambien F, Corvol P, Soubrier F: An insertion/deletion polymorphism in the angiotensin 1-converting enzyme gene accounting for half the variance of serum enzyme levels. J Clin Invest. 86, 1990, 1343-1346.

5: Busjahn A, Knoblauch H, Knoblauch M, Bohlender J, Menz M, Faulhaber HD, Becker A, Schuster H, Luft FC: Angiotensin-converting enzyme and angiotensinogen gene polymorphisms, plasma levels, cardiac dimensions: A twin study. Hypertension. 29 [part 2], 1997, 165-170.

6: Livak KJ: Allelic discrimination using fluorogenic probes and the 5´ nuclease assay. Genet Anal. 14, 1999, 143-149.

7: Cusi A, Barlassina C, Azzani T, Casari G, Citterio L, Devoto M, Glorioso N, Lanzani C, Manunta P, Righetti M, Rivera R, Stella P, Troffa C, Zagato L, Bianchi G: Polymorphisms of a-adducin and salt-sensitivity in patients with essential hypertension. Lancet. 349, 1997, 1353-1357.

8: Machin GA: Some causes of genotypic and phenotypic discordance in monozygotic twin pairs. Am J Med Genet. 61, 1996, 216-228.

9: Kyvik KO: Generalisability and assumptions of twin studiesSpector TD, Snieder H, MacGregor AJ: Advances in twin and sib-pair analysis. London. Greenwich Medical Media Ltd. 2000, 67-77.

10: Phillips DIW: Twin studies in medical research: can they tell us wether diseases are genetically determined?. Lancet. 341, 1993, 1009-1009.

11: Phillips DIW: Fetal programming or genes? Spector TD, Snieder H, MacGregor AJ: Advances in twin and sib-pair analysis. London. Greenwich Medical Media Ltd. 2000, 79-89.

12: Prescott CA, Johnson RC, McArdle JJ: Chorion type as a possible influence on the results and interpretation of twin study data. Twin Research. 2, 1999, 244-249.

13: Neale MC, Cardon LR: Methodology for genetic studies of twins and families. Dordrecht, Netherlands. Kluwer Academic Publishers. 1992, 496.

14: Jöreskog KG, Sörbom D: LISREL 8. Chicago IL. Scientific Software International Inc, 1993.

15: Neale MC: Mx: Statistical modeling4th edition. Richmond, VA. Department of Psychiatry, 1997.

16: Martin N, Boomsma D, Machin G: A twin-pronged attack on complex traits. Nat Genet. 17, 1997, 387-392.

17: S.A.G.E. Statistical Analysis for Genetic Epidemiology, Release 2.2. Case Western Reserve University. Cleveland, OH, 1994.

18: Kruglyak L, Lander ES: Complete Multipoint Sib-Pair Analysis of Qualitative and Quantitative Traits. Am J Hum Genet. 57, 1995, 439-454.

19: Haseman JK, Elston RC: The investigation of linkage between a quantitative trait and a marker locus. Behavior Genetics. 2, 1972, 3-19.

20: Fulker DW, Cherny SS, Sham PC, Hewitt JK: Combined linkage and association sib-pair analysis for quantitative traits. Am J Hum Genet. 64, 1999, 259-267.

21: Ewens WJ, Spielman RS: The transmission/disequilibrium test: history, subdivision, and admixture. Am J Hum Genet. 57, 1995, 455-464.

22: Allison DB: Transmission-disequilibrium tests for quantitative traits. Am J Hum Genet. 60, 1997, 676-690.

23: Busjahn A, Faulhaber HD, Viken RJ, Rose RJ, Luft FC: Genetic influence on blood pressure with the cold-pressor test: A twin study. J Hypertens. 14, 1996, 1195-1199.

24: Busjahn A, Aydin A, v.Treuenfels N, Faulhaber HD, Gohlke HR, Knoblauch H, Schuster H, Luft FC: Linkage but lack of association for blood pressure and

the α-adducin locus in normotensive twins. J Hypertens. 17, 1999, 1437-1441.

25: Nagy Z, Busjahn A, Bähring S, Faulhaber HD, Gohlke HR, Knoblauch H, Rosenthal M, Müller-Myhsok B, Schuster H, and Luft FC: Quantitative trait loci for blood pressure exist near the IGF-1, the liddle syndrome, the angiotensin II-receptor gene and the renin loci in man. JASN. 10, 1999, 1709-1716.

26: Busjahn A, Li GH, Faulhaber HD, Rosenthal M, Becker A, Jeschke E, Schuster H, Timmermann B, Hoehe MR, Luft FC: β-2 adrenergic receptor gene variations, blood pressure, and heart size in normal twins. Hypertension. 35, 2000, 555-560.

27: Li GH, Faulhaber HD, Rosenthal M, Schuster H, Jordan J, Timmermann B, Hoehe MR, Luft FC, Busjahn A: β-2 adrenergic receptor gene variations and blood pressure under stress in normal twins. Psychophysiology. (in press).

28: Tank J, Jordan J, Diedrich A, Stoffels M, Franke G, Faulhaber HD, Luft FC, Busjahn A: Genetic influences on baroreflex function in normal twins. Hypertension, 37, 2001, 907-910.

29: Busjahn A, Voss A, Knoblauch H, Knoblauch M, Jeschke E, Wessel N, Bohlender J, McCarron J, Faulhaber HD, Schuster H, Dietz R, Luft FC: Angiotensin-converting enzyme and angiotensinogen gene polymorphisms and heart rate variability in twins. Am J Cardiol. 81, 1998, 755-760.

30: Busjahn A, Knoblauch H, Faulhaber HD, Boeckel Th, Rosenthal M, Uhlmann R, Hoehe M, Schuster H, and Luft FC: The QT interval is linked to two long-qt syndrome loci in normal subjects. Circulation. 99, 1999, 3161-3164.

31: Assmann G, Schulte H: Relation of high density lipoprotein cholesterol and triglycerides to incidence of atherosclerotic coronary artery disease: the PROCAM experience. Am J Cardiol. 70, 1992, 733-737.

32: Knoblauch H, Busjahn A, Münter S, Nagy Z, Faulhaber HD, Schuster H, Luft FC: Heritability analysis of lipids and three gene loci in twins link the macrophage scavenger receptor to hdl cholesterol concentrations. Arteriosclerosis Thrombosis And Vascular Biology. 17, 1997, 2054-2060.

33: Knoblauch H, Busjahn A, Müller-Myhsok B, Faulhaber HD, Schuster H, Uhlmann R, Luft FC: Peroxisome proliferator activated receptor γ gene locus is related to body mass index and lipid values in normal subjectsArteriosclerosis. Thrombosis And Vascular Biology. 19, 1999, 2940-2944.

34: Costa PT, McCrae RR: Personality, stress, and coping: Some lessons from a decade of research. Markides KS, Cooper CL: Aging, stress, and health. New York. John Wiley & Sons Ltd. 1989, 269-285.

35: Theorell T: Life events and manifestations of ischemic heart disease: Epidemiological and psychological aspects. Psychosomatics. 34, 1980, 135-148.

36: Watson D, Hubbard B: Adaptational style and dispositional structure: Coping in the context of the Five-Factor model. J Pers. 64, 1996, 737-774.

37: Kendler KS, Kessler RC, Heath AC, Neale MC, Eaves LJ: Coping: A genetic epidemiological investigation. Psychol Med. 21, 1991, 337-346.

38: Mellins CA, Gatz M, Baker L: Childrens methods of coping with stress: A twin study of genetic and environmental influences. J Child Psychol Psychiat. 37, 1996, 721-730.

39: Busjahn A, Faulhaber HD, Freier K, Luft FC: Genetic and environmental influences on coping styles: A twin study. Psychosom Med. 61, 1999, 469-475.

40: Rapp JP, Wang SM, Dene H: A genetic polymorphism in the renin gene of Dahl rats cosegregate with blood pressure. Science. 243, 1989, 542-544.

41: Jeunemaitre X, Soubrier F, Kotelevsev YV, Lifton RP, Williams CS, Charru A, Hunt SC, Hopkins PN, Williams RR, Lalouel JM, Corvol P: Molecular basis of human essential hypertension: Role of angiotensinogen. Cell. 71, 1992, 168-180.

42: Caulfield M, Lavender P, Farrall M, Munroe P, Lawson M, Turner P, Clark AJ: Linkage of the angiotensinogen gene to essential hypertension. N Engl J Med. 330, 1994, 1629-1633.

43: Tiret L, Bonnardeaux A, Poirier O, Ricard S, Marques-Vidal P, Evans A, Arveiler D, Luc G, Kee F, Ducimetiere P: Synergistic effects of angiotensin-converting enzyme and angiotensin-II type 1 receptor gene polymorphisms on risk of myocardial infarction. Lancet. 344, 1994, 910-913.

44: Shimkets RA, Warnock DG, Bositis CM, Nelson-Williams C, Hansson JH, Schambelan M, Gill JR, Ulick S, Milora RV, Findling JW, Canessa CM, Rossier BC, Lifton RP: Liddle´s syndrome: Heritable human hypertension caused by mutations in the ß subunit of the epithelial sodium channel. Cell. 79, 1994, 407-414.

45: Schuster H, Wienker TF, Bähring S, Bilginturan N, Toka HR, Neitzel H, Jeschke E, Tokan O, Gilbert D, Lowe A, Ott J, Haller H, Luft FC: Severe autosomal dominant hypertension and brachydactyly in a unique Turkish kindred maps to human chromosome 12. Nat Genet. 13, 1996, 98-100.

46: Reymer PW, Gagne E, Groenemeyer BE, Zhang H, Forsyth I, Jansen H, Seidell JC, Kromhout D, Lie KE, Kastelein J: A lipoprotein lipase mutation (Asn291Ser) is associated with reduced HDL cholesterol levels in premature atherosclerosis. Nat Genet. 10, 1995, 28-33.

47: Williams RR, Hunt SC, Hopkins PN, Wu LL, Hasstedt SJ, Berry TD, Barlow GK, Stults BM, Schumacher MC, Ludwig EH: Genetic basis of familial dyslipidemia and hypertension: 15-year results from Utah. Am J Hypertens. 6, 1993, 319-327.

48: Andronico G, Mangano MT, Nardi E, Mule G, Piazza G, Cerasola G: Insulin-like growth factor 1 and sodium-lithium countertransport in essential hypertension and in hypertensive left ventricular hypertrophy. J Hypertens. 11, 1993, 1097-1101.

49: Bianchi G, Tripodi G, Casari G, Salardi S, Barber BR, Garcia R, Leoni P, Torielli L, Cusi D, Ferrandi M: Two point mutations within the adducin genes are involved in blood pressure variation. Proc Natl Acad Sci. USA, 91, 1994, 3999-4003.

50: Casari G, Barlassina C, Cusi D, Zagato L, Muirhead R, Righetti M, Nembri P, Amar K, Gatti M, Macciardi F: Association of the alpha-adducin locus with essential hypertension. Hypertension. 25, 1995, 320-326.

51: Skrabal F, Kotanko P, Luft FC: Minireview: inverse regulation of α-2 and β-2 adrenoceptors in salt-sensitive hypertension: A hypothesis. Life Sci. 45, 1989, 2061-2076.

52: Svetkey LP, Timmons PZ, Emovon O, Anderson NB, Preis L, Chen YT: Association of hypertension with the β-2 and α-2c10 adrenergic receptor genotype. Hypertension. 27, 1996, 1210-1215.

53: Svetkey LP, Chen YT, McKeown SP, Preis L, Wilson AFTI: Preliminary evidence of linkage of salt sensitivity in black Americans at the beta 2-adrenergic receptor locus. Hypertension. 29, 1997, 918-922.

54: Vincent GM: The molecular genetics of the long QT syndrome: genes causing fainting and sudden death. Annu Rev Med. 49, 1998, 263-274.

55: Busjahn A, Knoblauch H, Faulhaber HD, Aydin A, Uhlmann R, Tuomilehto J, Kaprio J, Jedrusik P, Januszewicz A, Strelau J, Schuster H, Luft FC, Müller-Myhsok B: A region on chromosome 3 is linked to dizygotic twinning. Nat Genet. 26, 2000, 398-399.

56: Busjahn A, Freier K, Faulhaber HD, Li G-H, Rosenthal M, Timmermann B, Schuster H, Hoehe MR, Luft FC: β2-adrenergic receptor gene variations and coping styles in normal twins. Behav Genet. (subm.).

57: Lynch M, Walsh J: Genetics and analysis of quantitative traits. Sunderland, MA. Sinauer Ass. Inc.1998, 980.

Danksagung

Die hier vorgelegten Studien waren nur realisierbar durch die Unterstützung, die mir von meiner Familie und Kollegen zuteil wurde.

Meine Eltern haben nicht nur meine genetische Ausstattung beigesteuert, sie haben vor allem die Umwelt zu verantworten, in der ein großer Teil meiner Entwicklung stattgefunden hat, und dafür gebührt Ihnen an dieser Stelle mein Dank.

Für Ihre Unterstützung und vor allem Toleranz möchte ich meiner Frau und meinen Kindern danken. Auch wenn ich hoffe, sie nicht wirklich vernachlässigt zu haben, bin ich mir nicht immer sicher.

Von den vielen Kollegen, die diese Arbeit durch ihre Mitwirkung ermöglicht haben, möchte ich in ganz besonderer Weise Prof. Friedrich C. Luft danken, ohne dessen Kenntnisse und Visionen über klinische Medizin und medizinische Forschung sowie die Unterstützung gegen erhebliche Widerstände die Bucher Zwillingsstudien undenkbar wären. In gleicher Weise hat auch Prof. Hans-Dieter Faulhaber durch die Mitgestaltung der Studie und die aktive Teilnahme an der Durchführung entscheidend zum Gelingen beigetragen, wofür ich ihm an dieser Stelle herzlich danken möchte.

Für ihre kollegiale Unterstützung danke ich weiterhin Dr. Hans Knoblauch sowie Prof. Herbert Schuster. Den meisten Kontakt mit den Zwillingen hatte sicher meine Assistentin, Frau Magda Rosenthal, und der größte Teil der DNA-Analysen wurde durch Frau Regina Uhlmann im genetischen Labor der Franz-Volhard-Klinik durchgeführt, beiden hier mein Dank für ihren wichtigen Beitrag zu den Studien.

Für ihre Bemühungen, mich in das Reich der mathematischen Analysen von Zwillingsdaten sowie das soziale Reich der Zwillingsforscher einzuführen, danke ich Richard J. Rose und Richard J. Viken von der Indiana University in Bloomington.

Für die Förderung meiner Arbeit durch ein Stipendium sowie Sachmittel danke ich der Deutschen Akademie der Naturforscher LEOPOLDINA, die in ihrer Bewertung des Potentials weitsichtiger war als die unbekannten Gutachter der DFG, die uns jede Förderung versagten.

Abschließend möchte ich meinem Betreuer bei der Erstellung dieser Habilitationsschrift, Prof. Jens Reich, danken.

Eidestattliche Erklärung

Hiermit erkläre ich eidesstattlich, dass die vorgelegte Habilitationsschrift ohne fremde Hilfe verfasst wurde und dass die Zusammenarbeit mit anderen Wissenschaftlern und technischen Hilfskräften sowie die Literatur vollständig angegeben wurden.

Berlin, den

Publikationen und Impaktfaktor (IF) 1999

- **Busjahn A**. Einsatz von Computerspielen zur Untersuchung von komplexen Zusammenhängen: Persönlichkeit - Umwelt - Reaktivität. *Software-Kurier* 2, 1989, 161-166. IF -
- Knust U, Homuth V, Richter Heinrich E, **Busjahn A**. Pilotstudie zur Langzeitwirkung einer kombinierten medikamentösen und psychophysiologisch orientierten Therapie bei Patienten mit einer essentiellen Hypertonie höheren Schweregrades. *Wiener klinische Wochenschrift* 103(6), 1991, 163-168. IF 0,6
- Homuth V, **Busjahn A**, Knust U, Jüpner A, Rückert E, Schneider M, Faulhaber HD, Luft FC. Blutdruckselbstmessung und 24-Stunden-ABDM bei milder Hypertonie in der Schwangerschaft. *Nieren- und Hochdruckkrankheiten* 22, 1993, 305-307. IF 0,2
- Homuth V, Jüpner A, **Busjahn A**, Rückert E, Reichert M, Faulhaber HD, Luft FC. Untersuchungen zur Klinik und Differentialtherapie der milden Hypertonie in der Schwangerschaft. *Zentralblatt für Gynäkologie* 116, 1994, 267-270. IF -
- **Busjahn A**, Faulhaber HD, Viken RJ, Rose RJ, Luft FC. Genetic Influence on Blood Pressure with the cold-pressor test: A Twin Study. *J Hypertens* 14, 1996, 1195-1199. IF 3,1
- **Busjahn A**, Gohlke HR, Homuth V. Querschnitts-Studie zur saisonalen Blutdruckvariation im ambulanten 24-Stunden-Blutdruckprofil. *Nieren- und Hochdruckkrankheiten* 24(6), 1996, 288-291. IF 0,2
- Schuster H, Wienker TF, Toka HR, Bähring S, Jeschke E, Toka O, **Busjahn A**, Hempel A, Tahlhammer C, Oelkers W, Kunze J, Bilginturan N, Haller H, Luft FC. Autosomal dominant hypertension and brachydactyly in a turkish kindred resembles essential hypertension. *Hypertension* 28(6), 1996, 1085-1092. IF 4,3
- Voss A, **Busjahn A**, Wessel N, Schurath R, Faulhaber HD, Luft FC, Dietz R. Familial and genetic influences on heart rate variability. *J Electrocardiol* 29 Supplement, 1996, 154-160. IF 0,3
- Becker A, **Busjahn A**, Faulhaber HD, Bähring S, Robertson J, Schuster H, Luft FC. Twin zygosity: automated determination with microsatellites. *J Reprod Medicine* 42(5), 1997, 260-266. IF 0,8

- Naraghi R, Schuster H, Toka HR, Baehring S, Toka O, Oztekin O, Bilginturan N, Knoblauch H, Wienker TF, **Busjahn A**, Haller H, Fahlbusch R, Luft FC. Neurovascular Compression at the Ventrolateral Medulla in Autosomal Dominant Hypertension and Brachydactyly. *Stroke* 28, 1997, 1749-1754. IF 4,9
- Haller H, Ziegler E-M, Homuth V, Drab M, Eichhorn J, Nagy S, **Busjahn A**, Vetter K, Luft FC. Endothelial Adhesion Molecules and Leukocyte Integrins in Preeclamptic Patients. *Hypertension* 29 [part 2], 1997, 291-296. IF 4,3
- **Busjahn A**, Knoblauch H, Knoblauch M, Bohlender J, Menz M, Faulhaber HD, Becker A, Schuster H, Luft FC. Angiotensin-Converting Enzyme and Angiotensinogen Gene Polymorphisms, Plasma Levels, Cardiac Dimensions: A Twin Study. *Hypertension* 29 [part 2], 1997, 165-170. IF 4,3
- Knoblauch H, **Busjahn A**, Munter S, Nagy Z, Hd F, Schuster H, FC L. Heritability analysis of lipids and three gene loci in twins link the macrophage scavenger receptor to hdl cholesterol concentrations. *Arteriosclerosis Thrombosis and Vascular Biology* 17(10), 1997, 2054-2060. IF 3,9
- **Busjahn A**. Zwillingsstudien zur Genetik des Herz-Kreislauf-Systems. *Nova Acta Leopoldina* Suppl.15, 1998, 105-114. IF -
- **Busjahn A**, Voss A, Knoblauch H, Knoblauch M, Jeschke E, Wessel N, Bohlender J, McCarron J, Faulhaber HD, Schuster H, Dietz R, Luft FC. Angiotensin-converting enzyme and angiotensinogen gene polymorphisms and heart rate variability in twins. *Am J Cardiol* 81(6), 1998, 755-760. IF 2,1
- Joubran R, Asmi M, **Busjahn A**, Vergopoulos A, Luft FC, Jouma M. Homozysteine levels and coronary heart disease in Syria. *J Cardiovascular Risk* 5, 1998, 257-261. IF -
- Haller H, Hempel A, Homuth V, Mandelkow A, **Busjahn A**, Maasch C, Drab M, Lindschau C, Jupner A, Vetter K, Dudenhausen J, Luft FC. Endothelial-cell permeability and protein kinase C in pre-eclampsia. *Lancet* 351(9107), 1998, 945-949. IF 11,8
- Müller DN, Fischli W, Clozel JP, Hilgers KF, Bohlender J, Menard J, **Busjahn A**, Ganten D, Luft FC. Local angiotensin II generation in the rat heart: role of renin uptake. *Circ Res 82*(1), 1998, 13-20. IF 8,0
- Pfeufer A, **Busjahn A**, Vergopoulos A, Knoblauch H, Urata H, Osterziel KJ, Menz M, Wienker TF, Faulhaber HD, Steinmetz A, Schuster H, Dietz R, Luft

FC. Chymase gene locus is not associated with myocardial infarction and is not linked to heart size or blood pressure. *Am J Cardiol* 82(8), 1998, 979-981.

- Schuster H, Toka O, Toka HR, **Busjahn A**, Oztekin O, Wienker TF, Bilginturan N, Bahring S, Skrabal F, Haller H, Luft FC. A cross-over medication trial for patients with autosomal- dominant hypertension with brachydactyly. *Kidney International* 53(1), 1998, 167-172. IF 3,8
- Thalhammer C, Balzuweit B, **Busjahn A**, Walter C, Luft FC, Haller H. Endothelial cell dysfunction and arterial wall hypertrophy are associated with disturbed carbohydrate metabolism in patients at risk for cardiovascular disease. *Arteriosclerosis Thrombosis and Vascular Biology* 19(5), 1999, 1173-1179. IF 3,9
- Timmermann B, Mo R, Luft FC, Gerdts E, **Busjahn A**, Omvik P, Li GH, Schuster H, Wienker TF, Hoehe MR, Lund-Johansen P. Beta-2 adrenoceptor genetic variation is associated with genetic predisposition to essential hypertension: The Bergen Blood Pressure Study. *Kidney Int* 53(6), 1998, 1455-1460. IF 3,8
- Nagy Z, **Busjahn A**, Bahring S, Faulhaber HD, Gohlke HR, Knoblauch H, Rosenthal M, Muller MB, Schuster H, Luft FC. Quantitative trait loci for blood pressure exist near the IGF-1, the Liddle syndrome, the angiotensin II-receptor gene and the renin loci in man. *J Am Soc Nephrol* 10(8), 1999, 1709-1716. IF 6,4
- **Busjahn A**, Aydin A, von-Treuenfels N, Faulhaber HD, Gohlke HR, Knoblauch H, Schuster H, Luft FC. Linkage but lack of association for blood pressure and the alpha-adducin locus in normotensive twins. *J Hypertens* 17(10), 1999, 1437-1441. IF 3,1
- **Busjahn A**, Faulhaber HD, Freier K, Luft FC. Genetic and environmental influences on coping styles: a twin study. *Psychosom Med* 61(4), 1999, 469-475. IF 3,0
- **Busjahn A**, Knoblauch H, Faulhaber HD, Boeckel T, Rosenthal M, Uhlmann R, Hoehe M, Schuster H, Luft FC. QT interval is linked to 2 long-QT syndrome loci in normal subjects. *Circulation* 99(24), 1999, 3161-3164. IF 9,2
- Knoblauch H, **Busjahn A**, Muller MB, Faulhaber HD, Schuster H, Uhlmann R, Luft FC. Peroxisome proliferator-activated receptor gamma gene locus is related to body mass index and lipid values in healthy nonobese subjects.

Arteriosclerosis Thrombosis and Vascular Biology 19(12), 1999, 2940-2944. IF 3,9

- Berger M, Schroder B, Daeschlein G, Schneider W, **Busjahn A**, Buchwalow I, Luft FC, Haller H. Chlamydia pneumoniae DNA in non-coronary atherosclerotic plaques and circulating leukocytes. *J Lab Clin Med* 136, 2000, 194-200. IF 2,0
- **Busjahn A**, Knoblauch H, Faulhaber HD, Aydin A, Uhlmann R, Tuomilehto J, Kaprio J, Jedrusik P, Januszewicz A, Strelau J, Schuster H, Luft FC, Müller-Myshsok B. A Region on Chromosome 3 is linked to dizygotic Twinning. *Nature Genet* 26, 2000, 398-399. IF 40,7
- **Busjahn A**, Li GH, Faulhaber HD, Rosenthal M, Becker A, Jeschke E, Schuster H, Timmermann B, Hoehe MR, Luft FC. beta-2 adrenergic receptor gene variations, blood pressure, and heart size in normal twins. *Hypertension* 35(2), 2000, 555-560. IF 4,3
- Kettritz R, Xu YX, Faass B, Klein JB, Muller EC, Otto A, **Busjahn A**, Luft FC, Haller H. TNF-alpha-mediated neutrophil apoptosis involves Ly-GDI, a Rho GTPase regulator. *J Leukoc Biol* 68(2), 2000, 277-283. IF 4,3
- Knoblauch H, Muller MB, **Busjahn A**, Ben-Avi L, Bähring S, Baron H, Heath SC, Uhlmann R, Faulhaber HD, Shpitzen S, Aydin A, Reshef A, Rosenthal M, Eliav O, Muhl A, Lowe A, Schurr D, Harats D, Jeschke E, Friedlander Y, Schuster H, Luft FC, Leitersdorf E. A cholesterol-lowering gene maps to chromosome 13q. *Am J Hum Genet* 66(1), 2000, 157-166. IF 10,9
- Pei WD, Baron H, Muller MB, Knoblauch H, Al-Yahyaee SA, Hui RT, Wu XG, Liu LS, **Busjahn A**, Luft FC, Schuster H. Support for linkage of familial combined hyperlipidemia to chromosome 1q21-q23 in Chinese and German families. *Clin Genet* 57(1), 2000, 29-34. IF0,9
- Szelestei T, Bähring S, Kovacs T, Vas T, Salamon C, **Busjahn A**, Luft FC, Nagy J. Association of a uteroglobin polymorphism with rate of progression in patients with IgA nephropathy. *Am J Kidney Dis* 36(3), 2000, 468-473. IF 3,1
- Tank J, Jordan J, Diedrich A, Stoffels M, Franke G, Faulhaber HD, Luft FC, **Busjahn A**: Genetic influences on baroreflex function in normal twins. *Hypertension*, 37, 2001, 907-910. IF 4,3

Anhang

Genetic influences on blood pressure with the cold-pressor test: a twin study

Andreas Busjahn, Hans-Dieter Faulhaber, Richard J. Viken*, Richard J. Rose* and Friedrich C. Luft

Objectives To determine the genetic and environmental contributions to resting blood pressure, the level of blood pressure during the cold-pressor test and the increase in blood pressure with the cold-pressor test in an adult cohort of normotensive twins.

Design and methods Ninety-one monozygotic and 41 dizygotic normal twin pairs were recruited by advertisement. The mean age was 34 ± 14 years (mean ± SD). Systolic blood pressure (SBP), diastolic blood pressure (DBP) and heart rate were measured continuously at the finger (using a Finapres device) and verified at the upper arm oscillometrically (using a Dinamap device) every minute. The cold-pressor test was conducted by immersing the non-dominant hand into cold (< 4°C) water for 2 min. Statistical analysis was performed by using the SPSS program; parameters of the quantitative genetic models were estimated by path-analysis techniques using the LISREL 8 program.

Results Heritability estimates of additive genetic effects were statistically significant for SBP and DBP but not for heart rate during rest and during the cold-pressor test. Furthermore, the path analysis indicated shared as well as specific genetic components both for the blood pressure level at rest and for that during the cold-pressor test. However, the genetic influences on the blood pressure level at rest and on the increase in blood pressure during the cold-pressor test (the blood pressure level during the cold-pressor test minus that during rest) were entirely independent of one another.

Conclusions A significant genetic covariation exists for SBP and DBP during rest and during the cold-pressor test, as well as a significant genetic variation that is specific to the cold-pressor stress condition. These findings suggest that different genes or sets of genes contribute to blood pressure regulation during rest and to blood pressure reactivity to cold-pressor stress.

Journal of Hypertension 1996, 14:1195–1199

Keywords: blood pressure, blood pressure genetics, psychological, stress, twin genetics

From the Franz Volhard Clinic and Max Delbrück Centre for Molecular Medicine, Virchow Klinikum, Humboldt University of Berlin, Germany, and the *Department of Psychology, Indiana University, Bloomington, Indiana, USA.

Sponsorship: This study was supported by a grant-in-aid to Andreas Busjahn from the Leopoldina Stiftung and by a grant-in-aid to Friedrich C. Luft from the Bundesministerium für Bildung und Forschung.

Request for reprints to Dr Andreas Busjahn, Franz Volhard Clinic, Wiltberg Straße 50, 13122 Berlin, Germany.

Received 8 January 1996 **Revised** 26 July 1996
Accepted 26 July 1996

Introduction

Weitz was the first to recognize the heritable nature of blood pressure [1] and was also the first to study hypertension in twins [2]. Since then, the highly efficient twin model has been used widely to study the heritability of blood pressure. Genetic variability of blood pressure is invariably found in twin studies [3]. Differences in the heritability estimates may be attributed to population differences, differences in protocols, various numbers of subjects and study design. In addition to the basal level of blood pressure, blood pressure responses to exogenous physical stress are also influenced by genetic variability [4]. The heritability estimates of these responses are variable also, perhaps because the populations differ or the tests employed are insufficiently standardized. Because stress and autonomic hyperactivity have been associated with the development of hypertension later in life [5,6], we studied basal blood pressure and heart rate under resting conditions, as well as under physical stress, in 132 pairs of monozygotic and dizygotic twins. We employed the cold-pressor test, since that test has been standardized well [7,8]. The cold-pressor test has been employed previously to test for genetic influences on blood pressure responses in twin studies; however, in these studies the effect of a shared environment was not estimated [9]. We were interested not only in determining the presence of genetic variability but also in examining the effect of a shared environment. We tested the hypothesis that the genetic factors influencing resting blood pressure also affect the absolute values observed during cold-pressor stress. Furthermore, we hypothesized that additional genetic factors, independent of those influencing the basal blood pressure level, affect the increase in blood pressure associated with cold-pressor stress.

Methods

Subjects and protocol

We recruited 132 pairs of twins (91 monozygotic and 41 dizygotic pairs) by print media advertisement to participate in studies involving blood pressure and blood pressure

reactivity to physical and mental stress. The subjects were all German Caucasians. They were recruited from various parts of Germany. In the present study we relied on the cold-pressor tests [7,8]. The protocol was approved by the Humboldt University's committee on the protection of human subjects and their written informed consent to participate was obtained from all of the subjects. Blood was obtained for the determination of zygosity. The zygosity was verified with the use of five polymerase chain reaction-amplified microsatellite markers and has been described in detail elsewhere (Becker A, *et al.*, unpublished data, 1995).

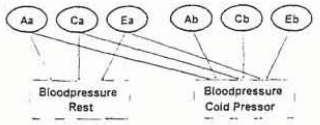
Blood pressure and heart rate were measured in the non-dominant arm by an automated oscillometric method (Dinamap; Tampa, Florida, USA) every minute, as well as continuously at the finger by the Finapres (Ohmeda, Louisville, Colorado, USA) blood pressure monitor. The latter device relies on the Penaz principle, according to which the blood pressure is determined in the middle finger of the right hand. Although the absolute values of this method may be influenced by various factors, intra-individual comparisons within one session are highly reliable [10]. The variability of blood pressure requires multiple measurements. Thus, all blood pressure data are based on the averaged continuous Finapres blood pressure values during 2 min. The dependent traits studied were the blood pressure level at rest, the blood pressure level during a cold-pressor test and the blood pressure increase during the cold-pressor test. The latter is the difference between the first two variables.

We relied on the cold-pressor test being an exogenous physical stressor in our studies. This test exhibits considerable standardization and reliability [11] and does not require the subject's cooperation as does the hand-gripping test. The sympathetic activation of the cold-pressor test in terms of increases in plasma noradrenaline values has been established [12]. The cold-pressor test has been used in earlier twin studies [13]. For the cold-pressor test, the subjects rested semirecumbent in a dental chair. The studies were conducted in a quiet, sound-proof room at 20°C. The subjects immersed their left hand into cold (< 4°C) water for 2 min after a 5 min rest period. For the resting blood pressure, we chose to use the averaged blood pressure values obtained continuously for 2 min 3 min after the cold-pressor test to avoid any possible pretest anxiety. The number of blood pressure values averaged ranged from 120 to 200 separate measurements.

Analytical methods

Statistical analysis was conducted using the SPSS program (SPSS Inc., Chicago, Illinois, USA). To test for differences in the mean level of the cardiovascular measures, Student's t-tests for independent groups were used. Parameters of the quantitative genetic models were estimated by path-analysis techniques using the LISREL 8 program developed by Jreskog and Srbom [14]. Analogously to a regression analysis, the variability of any given phenotype (P) within a population can be decomposed into genetic influences (A), environmental influences shared by the twins within a family (C) and random environment (E):

Fig. 1.



Bivariate path analysis model. Aa is a set of genes influencing both the basal and the stress level of blood pressure. Ab is a set of genes specific for blood pressure level during the cold-pressor test. C and E are environmental influences within and between families.

$$P = aA + cC + eE$$

with a, c and e the estimated relative influences. For monozygotes and dizygotes the covariances of their phenotypes are give by

$$r_{MZ} = a^2 + c^2 + e^2$$

$$r_{DZ} = 0.5a^2 + c^2 + e^2$$

respectively. Path analysis in twin studies can estimate additive and non-additive (dominance) components of genetic variability (estimated as h^2 and d^2) as well as two environmental influences, shared (c^2) and unshared (e^2) environmental influences [15]. These values estimate the relative amount of the variable's influence on interindividual differences up to a sum of unity. Genetic as well as environmental effects were estimated by the best fitting model selected by the χ^2-value. The LISREL 8 output also gives estimates of the quality of fit index, the adjusted quality of fit index and the Akaike information criterion. These estimates concurred with the results of the χ^2-analysis, so that we have elected not to present these estimates.

The hypothesis that different genes influence blood pressure during the resting state and during cold-pressor stress can be examined by using a bivariate path analysis [15]. The basic structure of the model, which assumes only additive genetic effects, is displayed in Figure 1. The path model includes two sets of genes, one influencing both resting and stress values (A_a), the second set of genes influencing only the stress values (A_b), two sets of shared environmental factors (C_a and C_b), and two sets of unshared environmental factors (E_a and E_b). In addition to comparing the absolute levels of blood pressure at rest and during the cold-pressor test, the bivariate model was applied to

Table 1 Demographic data on monozygotic and dizygotic twins

	Monozygotes	Dizygotes
Age (years)	34 ± 14	34 ± 12
Sex (male/female)	50/132	23/59
Height (cm)	168 ± 9	170 ± 8
Weight (kg)	66 ± 11	68 ± 13
Body mass index (kg/m²)	23 ± 4	23 ± 4
SBP during rest (mmHg)	128.7 ± 19	127.2 ± 18
DBP during rest (mmHg)	76.6 ± 12	75.6 ± 11
Heart rate during rest (beats/min)	71.6 ± 11	72.4 ± 10
SBP during cold-pressor test	140.4 ± 21	136.3 ± 19
SBP during cold-pressor test	84.2 ± 13	81.8 ± 12
Heart rate during cold-pressor test	76.7 ± 12	76.8 ± 11

Values are expressed as means ± SD. No differences were statistically significant. DBP, diastolic blood presusre; SBP, systolic blood pressure.

the resting level of blood pressure and to the blood pressure responses (ΔBP) to stress. Since the blood pressure at rest was not correlated to that during the cold-pressor stress in our subjects, we used the absolute differences (blood pressure during the cold-pressor test – blood pressure during rest) as a ΔBP value, rather than residualized change scores. This type of model tests the hypothesis that blood pressure during rest and ΔBP value during the cold-pressor stress share genetic variability.

We realize that twin studies have inherent limitations. Our model makes several simplified assumptions. The interactions between genetic and environmental factors cannot be tested with our methodology. Heritability estimates obtained from twin studies are generally greater than those found in family studies. The twin model assumes that the environmental effects in monozygotic and dizygotic twins are similar. This assumption is difficult to verify in individual instances and may not be invariably correct. Furthermore, heritability may conceivably change with age, although considerable stability in the heritability of blood pressure with age has been shown [9]. Since the age range in our study was relatively limited, we were not able to test any alterations in heritability influenced by age. However, environmental differences between the generations can result in lower heritability estimates in family studies.

Results

Demographic details of the twin subjects are given in Table 1. We studied 132 pairs of twins, 91 monozygotic and 41 dizygotic pairs. The monozygotic and dizygotic twins did not differ significantly with respect to geographical origin within Germany, age, gender distribution, height, weight, body mass index, blood pressure or heart rate during rest and during the cold-pressor test.

The effect of genetic variability determined by a conventional twin analysis is shown in Table 2. *P*-values for the significance of genetic effects were obtained by comparing χ^2-results between the model reported and the nested model without genetic influence. We were not able to identify any dominance effects. Thus, we present only the estimates for additive genetic effects. We were able to detect the effect of significant genetic variability on all the variables shown with the exception of heart rate. Thus, both resting systolic blood pressure (SBP) and diastolic blood pressure (DBP) during the cold-pressor test were influenced by genetic variability. A shared environment within the families had no significant influence on family resemblance in blood pressure, but it did on that in heart rate.

To test our hypothesis of common and specific genetic factors, the bivariate path analysis was used. Just like in the univariate case, no influence of a shared environment was needed in order to fit the data. The results in Figure 2 indicate that, besides genetic factors regulating basal blood pressure levels, specific genes are involved in blood pressure regulation during a cold-pressor test. For the blood pressure increase, again a significant genetic influence could be shown (Table 3), which was independent from genetic components of resting blood pressure. We present the χ^2-values for the most parsimonious model reported and for the competing model without a specific genetic effect on the blood pressure increase.

Discussion

The important findings in this study were that both resting blood pressure and cold pressor-evoked changes in blood pressure were influenced by genetic variability. Furthermore, we found support for the notion that the genetic influences on resting blood pressure and on the blood pressure increase associated with the cold-pressor test are not the same. This finding implies that the genes influencing resting blood pressure and those influencing the response to this physical stressor are different. This notion makes sense physiologically and has considerable implications for studies of molecular genetics. Different sets of candidate genes would come under consideration for study in terms of resting and stress-induced blood pressure. The analysis of covariance matrices for the two situations (resting blood pressure and blood pressure during a cold-pressor test) for the dizygotic twins militates against the possibility that our findings can be explained in terms of a change in penetrance of single genes, but rather implies that new sets of genes are recruited. Different penetrance functions under different conditions (resting blood pressure and blood pressure during a cold-pressor test) would be expected to result in altered heritability estimates. The values we observed during rest and under the influence of the cold pressor did not differ significantly.

We selected the cold-pressor test as a stress response manoeuvre, because the test is simple and standard [7,8]. Carmelli *et al.* [9] studied 47 monozygotic and 54 dizygotic older male twins aged 59–69 years. The heritability estimates for resting SBP and DBP were 0.96 and 1.00,

Table 2 **Genetic variability in resting blood pressure and blood pressure during cold-pressor stress**

	Additive genetic effect	Shared environmental effect	Environmental effect	χ^2 (degrees of freedom	r_{MZ}	r_{DZ}	Significance of genetic effect
SBP during rest	0.53		0.47	3.77 (4)	0.54	0.33	$P<0.01$
DBP during rest	0.52		0.48	5.97 (4)	0.54	0.32	$P<0.01$
Heart rate during rest		0.54	0.46	3.22 (4)	0.58	0.45	NS
SBP during cold-pressor test	0.57		0.43	2.85 (4)	0.59	0.30	$P<0.01$
DBP during cold-pressor test	0.56		0.44	7.59 (4)	0.59	0.27	$P<0.01$
Heart rate during cold-pressor test	0.24	0.40	0.36	2.50 (3)	0.66	0.45	NS

DBP, diastolic blood pressure; SBP, systolic blood pressure; r_{MZ} and r_{DZ}, for monozygotes and dizygotes, respectively.

Table 3 **Common and specific genetic variability in resting blood pressure and blood pressure increases during cold-pressor stress**

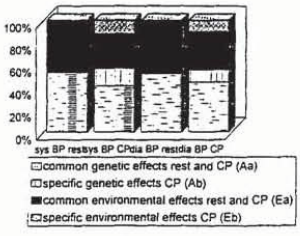
	Common genetic influence A_a	Specific genetic influence A_b	Common influence of unshared environment E_a	Specific influence of unshared environment E_b	χ^2 (degrees of freedom) for selected model	χ^2 (degrees of freedom) no specific genetic effect
SBP during rest	0.52		0.48			
ΔSBP during cold-pressor test	0	0.55	0.02	0.42	18.50 (15)	48.09 (15)
DBP during rest	0.51		0.48			
ΔDBP during cold-pressor test	0	0.52	0.01	0.46	21.03 (15)	47.31 (15)

DBP, diastolic blood pressure; SBP, systolic blood pressure; Δ, change.

respectively. With the cold-pressor test, these values remained significant at 0.70 and 0.38. Interestingly, when they examined the ΔBP with the cold pressor, neither the systolic nor the diastolic values were influenced significantly by genetic variability. These investigators restricted their analysis to analysis of variance methods and did not estimate the effects of a common environment. Ditto [16] examined a younger, mixed-sex sample of 100 twins aged 12–14 years. Their heritability estimates were 0.63 and 0.58 during rest. During the cold-pressor test, the SBP value was 0.38, whereas the DBP value differed between males (0.81) and females (0.22). The investigators also employed path-analysis techniques. Although they identified specific genetic factors for blood pressure values during rest and following cold-pressor stress, they were not able to prove any common genetic factors for basal blood pressure levels and cold-pressor stress.

Glass *et al.* [17] applied a different approach. They studied fathers and sons, which allowed them to examine genetic and environmental factors shared in each family simultaneously. They were not able to identify any correlations for the level of reactivity. Lovallo *et al.* [18] performed studies in normotensive and genetically hypertensive rats and were able to show that resting blood pressures and blood pressure values in response to physical stress could be bred in a divergent fashion. They concluded that different genes or different sets of genes were involved, which would be commensurate with the present findings.

Fig. 2.



Effect of genetic variance on systolic blood pressure (sys BP) and diastolic blood pressure (dia BP) during rest and during the cold-pressor test (CP).

The cold-pressor test is associated with higher levels of plasma noradrenaline and greater elevations in blood pressure than are tests of mental stress such as arithmetical problems [12]. Although some affective response is elicited by the cold-pressor test, it is primarily a physical stressor that involves the participant passively. In earlier twin studies, we were able to show that a variety of different regulatory systems controlling blood pressure are all influenced by genetic variability. These include electrolyte excretion, the renin–angiotensin–aldosterone system [19], the renal glomerular filtration rate [20] and plasma catecholamines [21]. Thus, the resting blood pressure is conceivably more influenced by long-term regulatory systems such as the renal pressure–natriuresis relationship, whereas the blood pressure response to the cold-pressor test involves acute sympathetic activation. These regulatory systems are controlled by different genes or sets of genes. Carmelli *et al.* [9] speculated earlier that the genes regulating the responses to mental arithmetic and those influencing the cold-pressor response are likely to be different.

We were surprised to find no shared environmental effect

on blood pressure under any conditions in our study. Interestingly, Ditto [16] also found shared environmental effects in their twin study only under the condition of psychological stress. Under resting conditions and during the cold-pressor test, no shared environmental effects were identified in their study. We suggest that a shared environment may be more important to psychologically influenced responses than it is to physically mediated effects.

We believe that our data lend support to our hypothesis regarding the genetic influences on blood pressure regulation at rest and under physical stress. We showed that genetic influences on cardiovascular regulation during cold-pressor stress can be quantified. Although the intraclass correlations we found were similar to those reported for older twins [9], the similarity in dizygotic twins was greater than had earlier been reported. The heritability estimates are not directly comparable, since a different method of calculation was used. The heritability of 1.0 reported by Carmelli *et al.* [9] would require exact concordance in monozygotic twins and precisely one-half concordance in dizygotic twins to appear as such in the path analysis. We were able to show specific genetic influences for our task analysis, which is similar to the results of Ditto [16]. However, we not only found specific genetic factors for the cold-pressor responses but also were able to quantify the contributions of genetic influences during rest and during stress. When sufficient numbers of monozygotic and dizygotic twins are available, we will be in a position to analyse gender-related differences. Moreover, we will be able to test polymorphisms in candidate genes using the twin model by relying specifically on the concordant and discordant responses of dizygotic twins and by testing for concordance or discordance at candidate gene loci [22]. The twin model was one of the first techniques used to investigate the genetics of hypertension. We suggest that the twin model will render further important contributions.

References

1 Weitz W: **Zur Ätiologie der genuinen oder vasculären Hypertension.** *Z Klin Med* 1923, **96**:150–181.
2 Weitz W: **Studien an eineiigen Zwillingen.** *Z Klin Med* 1925, **101**: 115–135.
3 Ward R: **Familial aggregation and genetic epidemiology of blood pressure.** In *Hypertension: Pathophysiology, Diagnosis, and Management.* Edited by Laragh JH, Brenner BM. New York: Raven Press Ltd; 1990: 81–99.
4 Rose RJ: **Genes, stress, and cardiovascular reactivity.** In *Individual Differences in Cardiovascular Response to Stress.* Edited by Turner JR, Sherwood A, Light KC. New York: Plenum Press; 1992:87–102.
5 Julius S, Johnson EH: **Stress, autonomic hyperactivity and essential hypertension: an enigma.** *J Hypertens* 1985, **3 (suppl 4)**:S11–S17.
6 Manuck St B: **Cardiovascular reactivity in cardiovascular disease: once more into the breach.** *Int J Behav Med* 1994, **1**:4–31.
7 Saab PG, Llabre MM, Hurwitz BE, Schneiderman N, Wohlgemuth W, Durel LA, *et al.*: **The cold pressor test: vascular and myocardial response patterns and their stability.** *Psychophysiology* 1993, **30**:366–373.
8 Sherwood A, Turner JR: **A conceptual and methodological overview of cardiovascular reactivity research.** In *Individual Differences in Cardiovascular Response to Stress.* Edited by Turner JR, Sherwood A, Light KC. New York: Plenum Press; 1992:3–32.
9 Carmelli D, Ward MM, Reed T, Grim CE, Harshfield GA, Gabsitz RR: **Genetic effects on cardiovascular responses to cold and mental activity in late adulthood.** *Am J Hypertens* 1991, 4:239–244.
10 Bos WJW, Imholz BPM, van Goudoever J, Weseling KH, van Montfrans GA: **The reliability of noninvasive continuous finger blood pressure measurement in patients with both hypertension and vascular disease.** *Am J Hypertens* 1992, **5**:529–535.
11 Mathias CJ, Bannister R: **Investigation of autonomic disorders.** In *Autonomic Failure.* Third Edition. Edited by Bannister R, Mathias CJ. Oxford: Oxford University Press; 1993:266.
12 LeBlanc T, Cote T, Jobin M: **Plasma catecholamines and cardiovascular responses to cold and mental activity.** *J Appl Physiol* 1979, **47**: 1207–1211.
13 McIlhany ML, Shaffer JW, Hines EA Jr: **The heritability of blood pressure: an investigation of 200 pairs of twins using the cold pressor test.** *Johns Hopkins Med J* 1975, **132**:57–64.
14 Jöreskog KG, Sörbom D: *LISREL 8.* Scientific Software International Inc. 11525 East 53rd Street, Suite 640, Chicago, IL 60615, USA, 1993.
15 Neale MC, Cardon LR: *Methodology for genetic studies of twins and families.* Dordrecht, Netherlands: Kluwer Academic Publishers; 1992:496.
16 Ditto B: **Familial influences on heart rate, blood pressure and self-reported anxiety responses to stress: results from 100 twin pairs.** *Psychophysiology* 1993, **30**:635–645.
17 Glass JC, McKinney ME, Hofshire PJ, Fedorko S: **Cardiovascular reactivity to stress: an examination of familial trends.** *Int J Psychophysiol* 1990, **9**:1–11.
18 Lovallo WR, Wilson MF: **A biobehavioral model of hypertension development.** In *Individual Differences in Cardiovascular Responses to Stress.* Edited by Sherwood A, Light KC. New York: Plenum Press; 1992: 265–180.
19 Grim CE, Miller JZ, Luft FC, Christian JC, Weinberger MH: **Genetic influence on renin, aldosterone, and the renal excretion of sodium and potassium following volume expansion and contraction in normal man** *Hypertension* 1979, 1:583–590.
20 Luft FC, Fineberg NS, Miller JZ, Rankin LI, Grim CE, Weinberger MH: **The effects of age, race and heredity on glomerular filtration rate in normal man.** *Am J Med Sci* 1980, **279**:15–24.
21 Miller JZ, Luft FC, Grim CE, Henry DP, Christian JC, Weinberger MH: **Genetic influences on plasma and urinary norepinephrine following volume expansion and contraction in normal man.** *J Clin Endocrinol Metab* 1980, **50**:219–222.
22 Morrison NA, Cheng Qi J, Tokita A, Kelly PJ, Crofts L, Nguyen TV, *et al.*: **Prediction of bone density from vitamin D receptor alleles.** *Nature* 1994, **367**:284–287.

Reprinted from THE JOURNAL OF REPRODUCTIVE MEDICINE
Vol. 42 No. 5, May 1997

Twin Zygosity

Automated Determination with Microsatellites

Albert Becker, M.S., Andreas Busjahn, Ph.D., Hans-Dieter Faulhaber, M.D., Sylvia Bähring, Ph.D., James Robertson, Ph.D., Herbert Schuster, M.D., and Friedrich C. Luft, M.D.

OBJECTIVE: Twin zygosity determinations can be performed with anthropologic, serologic and genetic markers; however, these methods are more than occasionally inefficient, often expensive and sometimes inaccurate. We used microsatellites as DNA markers and developed a largely automated, rapid and efficient method of determining zygosity.

STUDY DESIGN: We used five highly polymorphic short tandem repeat loci, coamplified by polymerase chain reaction (PCR) using fluorescence-labeled primers. Thirty-six samples were simultaneously analyzed by electrophoresis and laser detection. The PCR products were sized by automated fragment analysis.

RESULTS: We typed 132 pairs of monozygotic (MZ) and dizygotic (DZ) twins. With five markers, the probability that any twin pair was MZ if all markers were concordant was 99%.

CONCLUSION: This method is a rapid and reliable approach to zygosity detection. (J Reprod Med 1997;42: 260–266)

Keywords: microsatellite markers, zygote, DNA, twins.

Introduction

Twins and their families are frequently interested in zygosity for various reasons. Zygosity determination is a prerequisite of every twin study.[1] Numerous methods have been developed over the years, including anthropologic differences and similarities, blood groups, enzyme isoforms and DNA markers. More recently, highly polymorphic DNA microsatellite markers, also termed "short tandem repeats," have been described. These are a subgroup of variable numbers of tandem repeat (VNTR) markers.[2-4] The various alleles are easy to identify using the polymerase chain reaction (PCR), with single-copy primers flanking the polymorphic region. Allele determinations can be automated with fluorescence-labeled oligonucleotides and a DNA sequencer. We used five microsatellites—THO1, TPOX, FES/FPS, F13A1 and FGA—to develop an automated, two-step procedure.[5-8]

[As described here, for zygosity determination,] DNA fingerprinting using multilocus minisatellite DNA probes is an alternative approach.

Materials and Methods

We studied 132 pairs of normal MZ and DZ twins. Ninety-one pairs proved to be MZ and 41 were DZ. Twins were recruited by advertisements to partici-

From the Franz-Volhard Clinic, Max Delbrück Center for Molecular Medicine, Virchow Klinikum, Humboldt University, Berlin, Germany.

This study was supported by a grant-in-aid to Andreas Busjahn from the Leopoldina Stiftung and to Friedrich C. Luft from the Bundesministerium für Bildung und Forschung.

Address reprint requests to: Friedrich C. Luft, M.D., Franz-Volhard Clinic, Wiltbergstrasse 50, 13122 Berlin, Germany.

0024-7758/97/4205-0260/$1.50/0

pate in studies examining the effect of genetic variance on cardiovascular regulation. The mean age was 34 ± 14 (SD) years. Written, informed consent was obtained from all participants after the studies had been approved by Humboldt University's committee for the protection of human subjects.

Amplification Conditions

Total genomic DNA was extracted from leukocytes of the probands using a Genomix DNA Extraction System (Talent, Trieste, Italy). Primer sequences are shown in Table I. The oligonucleotides were synthesized, and fluorescent dyes FAM (540 nm) and JOE (560 nm) (Applied Biosystems, Foster City, California) were attached. The microsatellite loci were amplified using standard PCR conditions, as described elsewhere.[9] The samples were processed in a Gene Amp 9600 thermal cycler (Perkin Elmer, Foster City, California) programmed for 5.0 minutes of denaturing at 95°C, followed by 30 cycles of 15 seconds of denaturing at 94°C, a 2.0-minute ramp to annealing at 54°C for 30 seconds, and a 30-second extension at 72°C. Markers TPOX, THO1, FES/FPS and F13A1 were amplified as a multiplex and FGA as a single-PCR reaction under identical PCR conditions. Products were pooled before electrophoresis.

Electrophoresis and Automated Product Analysis

Internal lane size standard Genescan-500 (Applied Biosystems) labeled with red fluorescence dye ROX (610 nm) was used. This size standard consists of DNA fragments of 100, 140, 150, 160, 200, 250 and 300 base pairs in the size range. For gel analysis, 0.5 µL of the size standard, 1 µL, of the PCR products, 0.2 µL loading buffer (blue dextran, 50 mg/mL; EDTA, 25 mM) (Applied Biosystems) and 2 µL formamide were combined and heated to 95°C for 3 minutes before gel loading. A 24-cm, 6% polyacrylamide (Bio-Rad Laboratories, Hercules, California) gel was used for vertical electrophoresis in a 373 DNA Sequencer (Applied Biosystems). Thirty-six samples were loaded per gel, and electrophoresis was performed for eight hours at 30 W. The 672 Genescan and Genotyper software (Applied Biosystems) was used for detection, sizing and allele determination of PCR-amplified DNA fragments according to standard fragments.[10,11]

The method we applied, with fluorescence-labeled PCR primers and automated analysis technology, is similar to that used for forensic purposes.

Statistical Analysis

Allele frequencies in the twins were determined by gene counting from genotype data and compared with allele frequencies determined in a random white population of 100 unrelated individuals. To provide statistical evidence, the probability of a DZ twin pair was calculated to be concordant for the five genetic markers. This calculation was dependent on the number of alleles and their population frequencies.[12] The probability that a DZ twin is concordant for a specific genetic marker is:

$$P(\text{conc/DZ}) = \left[\left[1 + \sum_{i=1}^{N} p_i^2\right]^2 + \sum_{i \neq j}^{N} (p_i p_j)^2\right] /4,$$

Table I *Primer Sequences, Chromosome Locations and Fluorescent Dyes for Primer Labeling of the Five Tetranucleotide Repeat Markers—TPOX, FGA, F13A1, THO1 and FES/FPS*

Marker	Primer sequence	Chromosome	Fluorescent dye
TPOX	5'ACT GGC ACA GAA CAG GCA CTT AGG3' 5'GGA GGA ACT GGG AAC CAC ACA GGT3'	2 p	JOE
FGA	5'CTT CTC AGA TCC TCT GAC AC3' 5'CCA TAG GTT TTG AAC TCA CAG3'	4 q	FAM
F13A1	5'ATG CCA TGC AGA TTA GAA A3' 5'GAG GTT GCA CTC CAG CCT TT3'	6 p	JOE
THO1	5'GTG GGC TGA AAA GCT CCC GAT TAT3' 5'ATT CAA AGG GTA TCT GGG CTC TGG3'	11	FAM
FES/FPS	5'GTA GTC CCA GCT ACT TGG CTA CTC3' 5'GCT TGT TAA TTC ATG TAG GGA AGG C3'	15 q	FAM

where p_i is the frequency of the i^{th} allele in a system containing N alleles.[13] If the markers are independent, the *combined* probability that a DZ twin pair will be the same sex and concordant for all markers is:

Table II *Allele Frequencies of Markers TPOX, FGA, F13A1, THO1 and FES/FPS in a Random White Population, with Results from 100 Individuals*

Marker	Allele sizes	Allele frequencies in random population	Allele frequencies in twins
TPOX	157	—	0.002
	153	0.060	0.025
	149	0.260	0.281
	145	0.060	0.061
	141	0.055	0.104
	137	0.570	0.527
FGA	294	0.004	0.004
	290	0.016	0.021
	288	—	0.002
	286	0.082	0.070
	284	—	0.002
	282	0.131	0.121
	280	0.004	—
	278	0.143	0.135
	276	0.008	0.010
	274	0.238	0.197
	270	0.176	0.191
	268	0.004	0.006
	266	0.123	0.156
	262	0.070	0.066
	260	—	0.008
	256	—	0.002
F13A1	247	0.006	—
	231	0.012	0.020
	227	0.025	0.006
	223	0.012	0.021
	219	0.006	0.004
	211	—	0.002
	195	0.296	0.357
	191	0.290	0.320
	187	0.185	0.174
	183	0.068	0.023
	181	0.099	0.072
THO 1	174	0.012	0.006
	173	0.383	0.334
	170	0.124	0.115
	166	0.105	0.121
	162	0.185	0.227
	158	0.191	0.195
	154	—	0.002
FES/FPS	230	0.037	0.029
	226	0.278	0.158
	222	0.407	0.500
	218	0.259	0.281
	214	0.006	0.008
	210	0.012	0.023

$$Pc = P(\text{concordant and same sexed/DZ}) = 0.5 \times P_{c1} \times P_{c2} \times ... \times P_{cx}.$$

Gender is treated as a marker with a concordance probability of approximately 0.5. Using the combined probability, the *a posteriori* probability of being MZ for twins who have the same gender and are concordant for all markers can be calculated using Bayes Theorem:

$$P(\text{MZ/conc and same sexed}) = \frac{1}{1 + (Q \times 0.5 \times p_{c1} \times ... \times p_{cx})}.$$

The value Q (1.5 in Germany) is the *a priori* ratio of DZ/MZ in the population.[14]

Results

Figure 1 shows single and coamplified PCR products of markers THO1, TPOX, FES/FPS and F13A1 after agarose gel electrophoresis. To accelerate the process, multiplex conditions were developed for four markers. In Figure 2 a gel image of the DNA fragments is shown after electrophoresis using the red-labeled size standard, the green-labeled TPOX and F13A1 markers, and the blue FES/FPS, FGA and THO1 markers. To demonstrate the multiplex approach, the markers were analyzed separately (lanes 2–5) and after multiplexing (lane 6). In lane 7 the multiplex PCR pooled with the PCR product of marker FGA was electrophoresed simultaneously. This procedure was performed for the entire twin series. An electropherogram based on electrophoresis data on two sets of twins is demonstrated in Figure 3. Markers from each person were run in the same lane on the gel. The electropherogram shows the fluorescence intensity of each band as it passes by the detection system. The numbers in boxes below the peaks give the calculated fragment sizes. Those twin pairs concordant for all markers were regarded as monozygous. The dizygous set of twins

Table III *Probability That a DZ Twin Pair Is Concordant for the Five Markers*

Marker locus	P(conc/DZ)	P(conc and same-sex/DZ) combined
TPOX	.505	.253
FGA	.337	.085
F13A1	.381	.032
THO1	.396	.013
FES/FPS	.445	.006

The combined probability that a DZ twin pair is concordant for all markers and same sex includes a factor of 0.5, which is the approximate frequency of same-sex twins.

in Figure 3 shows discordance for markers THO1 and F13A1. Figure 4 shows an example of a Genescan analysis gel file containing 170 genotypes on a single gel.

In Table II, allele frequencies are given for the random, white population and for the twin pairs. There was no significant difference between these two groups. Some alleles were detected only in twins but not in the random sample and vice versa. The cumulative probability for a pair of DZ twins of the same sex to be concordant for all markers and of same sex is given in Table III. This probability was found to be .006. Thus, the probability that a male or female twin pair was concordant for all markers was 99.11%.

Discussion

Traditionally, zygosity determinations have been performed as a stepwise procedure using serologic markers, elaborate blood typing, HLA typing or amylase isoenyzmes to identify at least one marker discordant in a given twin pair.[15] Since these approaches cannot be mutiplexed, they are time consuming and labor intensive. DNA fingerprinting using multilocus minisatellite DNA probes is an al-

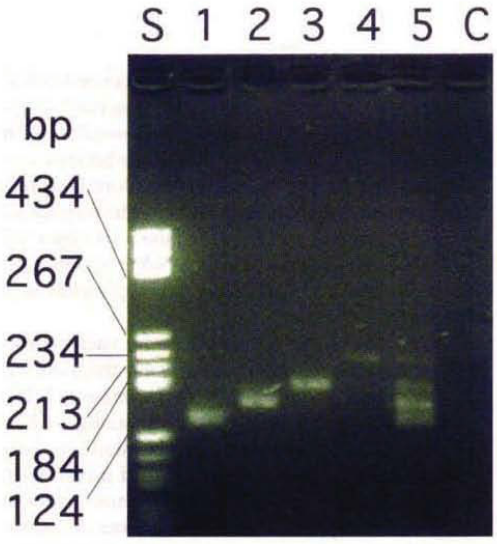


Figure 1 Agarose gel electrophoresis of PCR products. S = size standard, 1 = marker TPOX, 2 = marker THO1, 3 = marker F13A1, 4 = marker FES/FPS, 5 = multiplex PCR product of the four markers, C = negative control. Numbers give fragment sizes in base pairs.

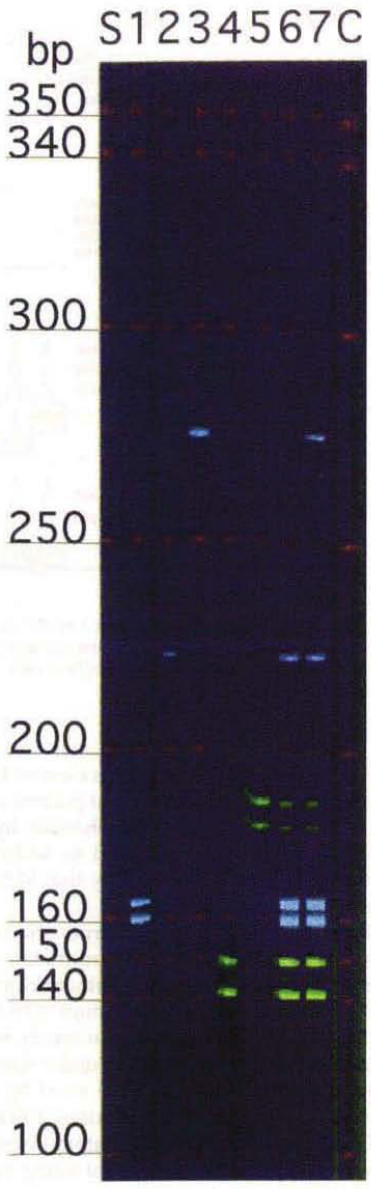


Figure 2 Genescan analysis gel file of electrophoresed single and multiplex amplified PCR products. The numbers give sizes in base pairs. S = size standard ladder Genescan ROX 500 (red), 1 = marker THO1 (blue), 2 = marker FES/FPS (blue), 3 = marker FGA (blue), 4 = marker TPOX (green), 5 = marker F13A1 (green), 6 = multiplex PCR, 7 = multiplex PCR pooled with marker FGA, and C = control.

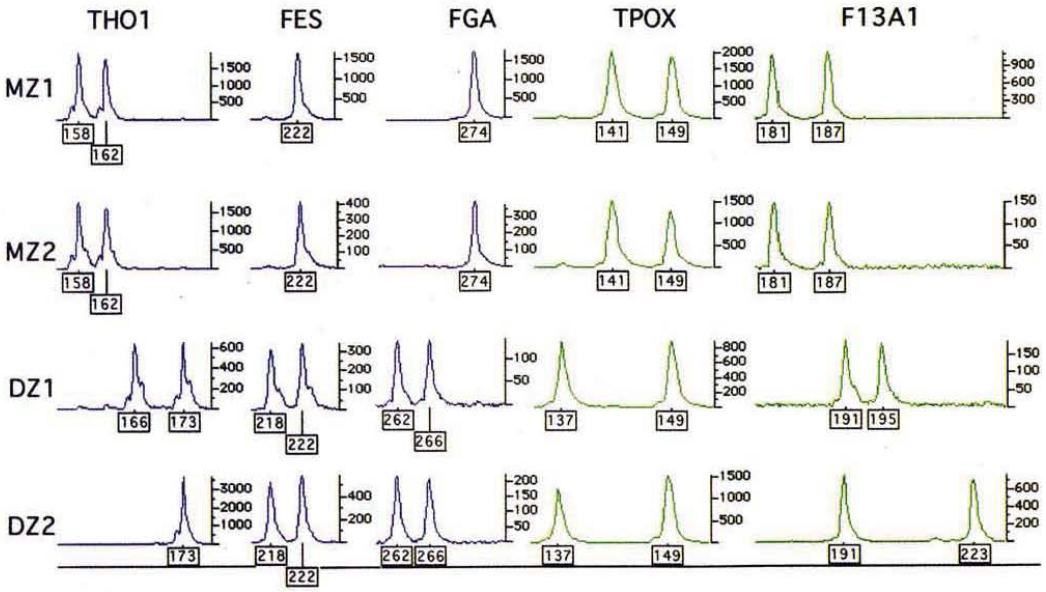


Figure 3 Electropherographic coanalysis of five markers for an MZ (1 and 2) and DZ (1 and 2) twin pair. Graphs are presented in the color of the primer labeling dyes. The calculated fragment sizes in base pairs can be found in boxes below the peaks. The numbers on the y-axis give the relative fluorescence intensity of the bands. MZ 1 and 2 are concordant for all markers, whereas DZ 1 and 2 are discordant for markers TPOX and F13A1.

ternative approach.[16,17] The DNA is cleaved by restriction enzymes, and the fragment pattern of the twin pair is compared after electrophoresis. Incomplete digestion of DNA may lead to additional bands; that results in the possibility that MZ twin pairs could be misclassified as DZ.

VNTR markers for zygosity determination were first described in combination with restriction fragment length polymorphism and Southern blot techniques.[18] Recently, zygosity determinations were performed with PCR microsatellite markers, which offer the advantage of amplification under standard PCR conditions.[19] Fragments were sized by electrophoresis on a standard, denaturing, polyacrylamide DNA sequencing gel, and autoradiographic analysis was applied. The fragment-sizing procedure with a very limited number of markers per gel lane for analysis is the rate-limiting step of this method. Furthermore, distinguishing homozygous from heterozygous individuals whose allele size differs by only two nucleotide bases is difficult because of overlapping bands. This problem occurs frequently if dinucleotide repeat markers are used. The method we applied, with fluorescence-labeled PCR primers and automated analysis technology, is similar to that used for forensic purposes.[20,21] PCR products that are in the same size range become distinguishable by using two different-colored fluorescent dyes for labeling. No signal interference of fragments running in the same lane was observed (Figure 3). Thus, the number of DNA fragments analyzed per lane and gel during electrophoresis was increased.

We analyzed the five markers from each person in the same lane; 36 samples were electrophoresed and analyzed within 8 hours on the same gel. We enhanced the accuracy of fragment sizing in two ways. First, the fragments of the size standard ladder were labeled with a third dye and mixed with every sample for coelectrophoresis. Thus, errors in sizing caused by lane-to-lane differences in migration of the fragments were eliminated. Second, the use of fluorescence labels allowed the simultaneous analysis of loci signals varying greatly in intensity. As compared to autoradiographic methods, fluorescent labels are linear over a greater range of sig-

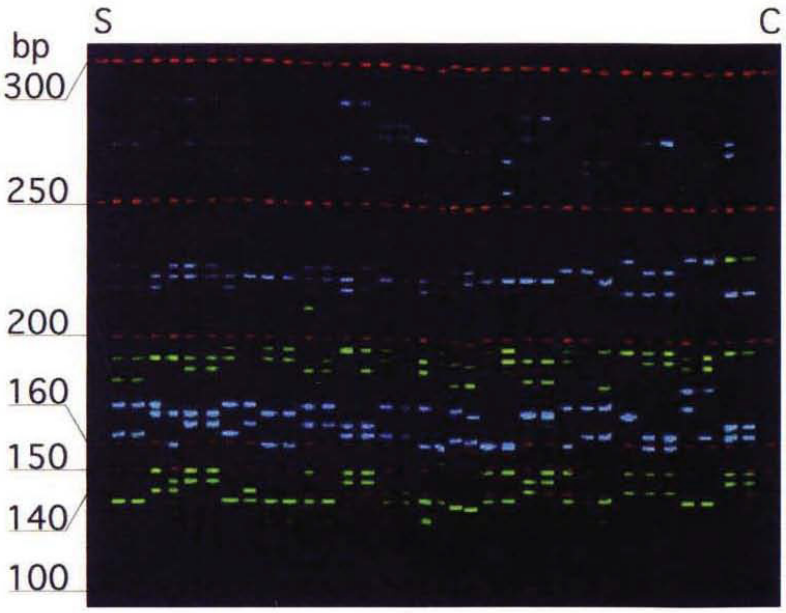


Figure 4 Genescan analysis gel file of electrophoresed PCR products of 17 twin pairs. PCR products of related individuals were run in neighboring lanes of the gel. MZ twins show identical; DZ twins show different bands. Markers TPOX and FF13A1 are presented in green and THO1, FGA and FES/FPS in blue. Size standard is labeled in red. S = size standard, C = negative control.

nal intensity. In addition, the software compensates for intensity variation. The electropherographic coanalysis shown in Figure 3 is an example. Although the fluorescence intensity of the bands varied across a wide range (y-axis), the resulting peaks were sufficient for the sizing procedure.

To obtain sufficient statistical power, we combined data from the multiplex PCR with that of a fifth marker. In future zygosity determinations, we will establish a one-tube, one-step PCR reaction. As the number of microsatellite marker loci amplified under the same PCR conditions increases, the capacity for analyzing multiple markers simultaneously will become easier. As more fluorescent dyes become available, there will be potential for increasing the efficiency of the method even further. Also, the power of PCR is such that extremely small samples are sufficient. For instance, we are currently performing zygosity testing on saliva rather than blood. Finally, our approach allows additional flexibility. Since we are particularly interested in molecular genetic studies, we have also used markers lying within or in proximity to genes of specific interest. We are currently focusing on genes involved in lipid metabolism; however, with currently available markers, any gene locus lends itself to that approach. In that way we can combine our genotyping efforts with our zygosity determinations in a single procedure.

Acknowledgments

The DNA for the TPOX marker was kindly provided by Dr. Jean-Paul Moisan, Laboratoire de Genetique Moleculaire, Centre Hospitalier Regional et Universitaire, Nantes, France. We are grateful to Dr. James M. Robertson, Applied Biosystems, Foster City, California, for his generous assistance. In part this work fulfilled the requirements for Albert Becker's M.D. degree.

References

1. Gedda L: Twins in History and Science. Springfield, Illinois, Charles C Thomas, 1954
2. Nakamura Y, Leppert M, O'Connell P, et al: Variable number of tandem repeat (VNTR) markers for human gene mapping. Science 1987;235:1616–1622
3. Weber JL, May PE: Abundant class of human DNA polymorphisms which can be typed using the PCR. Am J Hum Genet 1989;44:388–396
4. Litt M, Luty JA: A hypervariable microsatellite revealed by in vitro amplification of a dinucleotide repeat within the cardiac muscle actin gene. Am J Hum Genet 1989;44: 397–401

5. Hammond HA, Jin L, Zhong Y, et al: Evaluation of 13 short tandem repeat loci for use in personal identification applications. Am J Hum Genet 1994;55:175–189
6. Huang NE, Schumm J, Budowle B: Chinese population data on three tetrameric short tandem repeat loci—HUMTHO1, TPOX, and CSF1PO—derived using multiplex PCR and manual typing. Forensic Sci Int 1995;71:131–136
7. Polymeropoulos MH, Rath DS, Xiao H, et al: Tetranucleotide repeat polymorphism at the human coagulation factor XIII A subunit gene (F13A1). Nucleic Acids Res 1991;19:4306
8. Mills KA, Even D, Murray JC: Tetranucleotide repeat polymorphism at the human alpha fibrinogen locus (FGA). Hum Mol Genet 1992;1:779
9. Saiki RK, Gelfand DH, Stoffel S, et al: Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science 1988;239:487–491
10. Ziegle JS, Su Y, Corcoran KP, et al: Application of automated DNA sizing technology for genotyping microsatellite loci. Genomics 1992;14:1026–1031
11. Reed PW, Davies JL, Copeman JB, et al: Chromosome-πspecific microsatellite sets for fluorescence-based, semiautomated genome mapping. Nature Genet 1994;7:390–395
12. Smith SM, Penrose LS: Monozygotic and dizygotic twin diagnosis. Ann Hum Genet 1955;19:273–289
13. Selvin S: Efficiency of genetic systems for diagnosis of twin zygosity. Acta Genet Med Gemellol 1977;26:81–82
14. Krüger J, Propping P: Twinning frequencies in Baden Württemberg according to maternal age and parity from 1955–1972. Acta Genet Med Gemellol 1976;25:36–40
15. Vogel F, Motulsky AG: Diagnosis of zygosity. *In* Human Genetics. Edited by F Vogel, AG Motulsky. Berlin, Springer Verlag, 1986, pp 669–675
16. Jones L, Thein SL, Jeffreys AJ, et al: Identical twin marrow transplantation for 5 patients with chronic myeloid leukaemia: Role of DNA fingerprinting to confirm monozygosity in 3 cases. Eur J Haematol 1987;39:144–147
17. Machin GA: Definitive methods of zygosity determination in twins: Relevance to problems in the biology of twinning. Acta Genet Med Gemellol 1990;39:459–471
18. Akane A, Matsubara K, Shiono H, et al: Diagnosis of twin zygosity by hypervariable RFLP markers. Am J Med Genet 1991;41:96–98
19. Erdmann J, Nöthen MM, Stratmann M, et al: The use of microsatellites in zygosity diagnosis of twins. Acta Genet Med Gemellol 1993;42:45–51
20. Sullivan KM, Pope S, Gill P, et al: Automated DNA profiling by fluorescent labeling of PCR products. PCR Methods Appl 1992;2:34–40
21. Fregeau CJ, Fourney RM: DNA typing with fluorescently tagged short tandem repeats: A sensitive and accurate approach to human identification. Biotechniques 1993;15: 100–119

Angiotensin-Converting Enzyme and Angiotensinogen Gene Polymorphisms, Plasma Levels, Cardiac Dimensions

A Twin Study

Andreas Busjahn, Hans Knoblauch, Margit Knoblauch, Jürgen Bohlender, Marianne Menz, Hans-Dieter Faulhaber, Albert Becker, Herbert Schuster, Friedrich C. Luft

Abstract We tested the hypotheses that angiotensin-converting enzyme insertion/deletion (I/D) and angiotensinogen 235 methionine/threonine (M/T) substitution gene polymorphisms influence angiotensin-converting enzyme and angiotensinogen serum concentrations and cardiac dimensions in 91 monozygotic and 41 dizygotic twin pairs. Cardiac dimensions were determined echocardiographically. Angiotensin-converting enzyme levels were 24±11, 43±18, and 58±24 U/L for the II, ID, and DD genotypes, respectively ($P<.01$). Posterior wall thickness was 8.1±1.3, 8.6±1.7, and 8.9±1.9 mm for these genotypes ($P<.05$). Angiotensin-converting enzyme levels were correlated with posterior wall thickness ($r=.15$, $P<.05$). The intrapair differences in angiotensin converting enzyme levels for monozygotic, concordant dizygotic, and discordant dizygotic twins were 1.36±1.6, 1.86±1.6, and 17.25±4.3 U/L, respectively. The angiotensinogen M/T genotypes exerted no influence on cardiac dimensions or on angiotensinogen concentrations. The additive genetic effect on angiotensin-converting enzyme levels (0.49), on posterior wall thickness (0.26), and on septum thickness (0.37) was significant ($P<.01$), although shared and nonshared environmental effects were also identified. Our data confirm the impressive effect that the angiotensin-converting enzyme D allele exerts on angiotensin-converting enzyme plasma levels. Furthermore, our data also suggest that the angiotensin-converting enzyme gene locus is primarily responsible for angiotensin-converting enzyme plasma levels. Our twin study also indicates that the angiotensin-converting enzyme gene locus is genetically linked to posterior wall thickness. The correlation between angiotensin-converting enzyme levels and posterior wall thickness suggests that this effect is exerted by angiotensin-converting enzyme. We were unable to demonstrate genetic linkage between the angiotensinogen gene locus and cardiac dimensions in this study. **(*Hypertension*. 1997;29[part 2]:165-170.)**

Key Words • genetics • twins • ACE polymorphisms • angiotensinogen polymorphisms • cardiac hypertrophy

A diallelic polymorphism in the ACE gene, characterized by a D or I allele in the 16th intron of the ACE gene, has been associated with differences in plasma ACE levels, as well as risk for myocardial infarction and cardiac hypertrophy.[1] Tiret et al[2] used evidence from combined segregation and linkage analysis and showed that the I allele was characterized by lower ACE levels. A similar association between the I and D alleles and ACE in monocytes has also been identified.[3] Cambien et al[4] relied on an association study, in which the DD genotype was associated with myocardial infarction in men with low risk. They found that in that group, ACE levels did not decrease with age and were higher in patients with the DD and ID genotypes than in control subjects. Schunkert et al[5] reported an excess homozygosity for the D allele among subjects with cardiac hypertrophy as assessed by electrocardiographic criteria. The D allele has also been associated with the severity of cardiac hypertrophy in patients with hypertrophic cardiomyopathy.[6] A second renin-angiotensin system gene associated with heart disease is the AGT gene. A 235 M/T substitution (T for M) has been associated with higher blood pressures[7,8] and higher AGT levels, at least in blacks. To our knowledge, no twin study has been done to test the relationship between the ACE D allele, ACE plasma levels, the AGT T allele, and AGT levels and cardiac dimensions. We conducted a study in monozygotic and dizygotic twin pairs to test these hypotheses.

From the Franz Volhard Clinic and Max Delbrück Center for Molecular Medicine, Virchow Klinikum, Humboldt University of Berlin; and the Department of Clinical Pharmacology, Klinikum Benjamin Franklin, Free University of Berlin (M.K.), Germany.

Correspondence to Friedrich C. Luft, Franz Volhard Clinic, Wiltberg Strasse 50, 13122 Berlin, Germany. E-mail fcluft@mdc.berlin.de.

Methods

Subjects and Protocol

We recruited 132 pairs of MZ (91 pairs) and DZ (41 pairs) of twins by print media advertisement to participate in studies involving blood pressure and blood pressure reactivity to physical, and mental stress. The protocol was approved by the University's ethical committee on the protection of human subjects and written informed consent was obtained from all participants. Venous blood was obtained for genomic DNA. Each participant underwent a medical history and physical examination. None had hypertension or any other chronic medical illness. Blood pressure and heart rate were measured in the nondominant arm under standardized conditions by an automated oscillometric method (Dinamap).

Echocardiography

M-mode and two-dimensional echocardiograms were recorded with patients in the left lateral decubitus position. M-mode tracings that were guided two-dimensionally were recorded from the short parasternal axis at the chordal level between the free edges of the mitral leaflets at the tips of the papillary muscles. Only tracings with optimal visualization of left ventricular interfaces

Selected Abbreviations and Acronyms

ACE = angiotensin-converting enzyme
AGT = angiotensinogen
Ang = angiotensin
D = deletion
I = insertion
M = methionine
T = threonine

were used. In our echocardiographic laboratory, the range of variability of observations by a single reader is 0 mm to 1.5 mm for the left ventricular dimensions and 0 mm to 0.5 mm for the wall thickness. Interventricular septal thickness and posterior wall thickness were measured in all patients according to the guidelines of the American Society of Echocardiography.[9]

Laboratory Methods

The zygosity was verified with the use of five highly polymorphic short tandem repeat polymerase chain reaction–amplified microsatellite markers, namely THO1, TPOX, FES/FPS, F13A1, and FGA.[10] We used fluorescent labeled primers in a multiplex automated genotyping system relying on a 373 DNA sequencer, 672 GENESCAN, and GENOTYPER software (all Applied Biosystems). The I/D polymorphism of the ACE gene was identified with the polymerase chain reaction using a set of oligonucleotide primers flanking the polymorphic site according to the method described by Rigat et al.[11] Allele specific oligonucleotide hybridization was used for the genotyping of AGT codons 174 and 235. Genomic DNA was subjected to 30 rounds of amplification using primers described elsewhere.[12] The resulting 354 base pair fragment was denatured, dot blotted in duplicate onto nitrocellulose, and then neutralized. The filters were subsequently hybridized to the appropriate ^{32}P-labeled oligonucleotides. ACE activity in plasma was determined by means of a synthetic substrate (FAPGG) as outlined elsewhere,[13] and plasma AGT was determined by radioimmunoassay.[14] Since oral contraceptives increase AGT concentrations, we included only AGT determinations from men and women not taking oral contraceptives in our calculations.

Analytical Techniques

Statistical analysis was conducted using the SPSS program. To test for differences in the mean level of the cardiovascular measures, *t* tests for independent groups were used. In addition to univariate methods, analysis of variance was employed as a true multivariate method. Parameters of the quantitative genetic models were estimated by path analysis techniques using the LISREL 8 program developed by Jöreskog and Sörbom.[15] Analogous to a regression analysis, the variability of any given phenotype (P) within a population can be decomposed in additive genetic influences (A), nonadditive genetic influences (D), environmental influences shared (common) by the twins within a family (C), and random environment (E):

$$(1) \qquad P=aA+dD+cC+eE$$

with a, c, and e as the estimated relative influence. For MZ and DZ, the covariance of their phenotype is given by:

$$rMZ=a^2+d^2+c^2+e^2 \text{ and } rDZ=0.5a^2+0.25d^2+c^2+e^2$$

Path analysis in twin studies can estimate additive and nonadditive (dominance) components of genetic variability (estimated as a^2 and d^2) as well as two environmental influences, shared (c^2), and nonshared environmental influences (e^2).[16] These values estimate the relative amount of the variable's influence on interindividual differences up to a sum of one. Genetic as well as environmental effects were estimated by the best fitting model as selected by the χ^2 value. The LISREL 8 output also gives estimates of the Goodness of Fit Index, the Adjusted Goodness of Fit Index, and the Akaike Information Criterion. These estimates concurred with the results of the χ^2 analysis, so that we have elected not to present these estimates.

Allele frequencies and standard binomial errors were determined by the gene counting method. Genotype distributions of all groups were checked for Hardy-Weinberg equilibrium and were compared to each other by χ^2 and likelihood ratio methods. Relative risk figures (odds ratio statistics) and their 95% confidence intervals were calculated by using the SPSS statistical program package. Allele-dose (codominant) effects were tested by linear regression.

Results

In Table 1 are shown the demographic data of 139 subjects displayed in terms of ACE genotype. Only one member of each twin pair was included for the association testing. Selecting either twin 1 or twin 2 did not influence the results. No differences in terms of age, gender, body dimensions, systolic blood pressure or heart rate are apparent. Diastolic blood pressure was actually slightly higher ($P<.1$) in subjects with at least one I allele than in subjects with the DD genotype. Significant differences ($P<.01$) between the genotypes were observed in ACE levels. Interestingly, the AGT concentrations were significantly lower in persons with the ACE DD genotype than in those with the II or ID genotype. Posterior wall thickness was greater in subjects with the DD genotype than in those with the II genotype. Since gender, age, body size, weight, BMI, sys-

TABLE 1. Demographic, Clinical, and Echocardiographic, and ACE and AGT Plasma Level Data in Groups With Different Ace Genotypes

Variables	II	ID	DD	*P*	Group Differences
No.	27	79	33		
Age, y	34±14	33±14	34±14	NS	
Sex, M/F	6/21	18/61	10/23	NS	
Height, cm	169±8	168±8	170±9	NS	
Weight, kg	66±9	66±13	69±14	NS	
BMI, kg/m^2	23±4	23±4	23±5	NS	
Systolic BP, mm Hg	123±17	123±15	123±14	NS	
Diastolic BP, mm Hg	75±10	73±11	70±10	NS	
HR, bpm	70±9	73±13	73±9	NS	
ACE level, U/L	25±11	44±18	57±25	<.01	II-ID, II-DD, ID-DD
AGT, mg Ang I/mL	1.33±0.35	1.31±0.33	1.18±28	<.05	II-DD, ID-DD
Posterior wall, mm	7.9±1.2	8.5±1.0	8.8±1.9	<.05	II-ID, II-DD
Septum, mm	8.5±1.1	8.7±1.2	8.9±1.2	NS	

BMI indicates body mass index; BP, blood pressure; and HR, heart rate. Values are mean±SD.

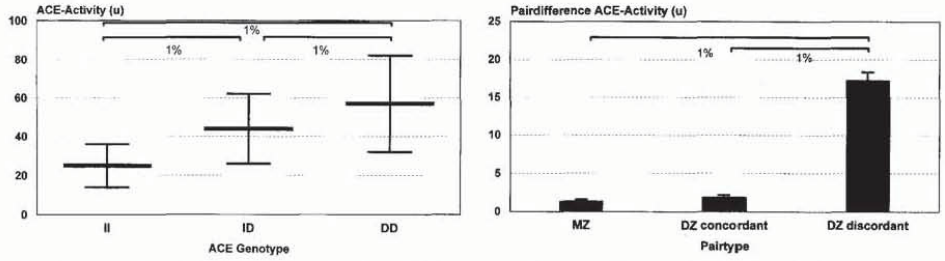


FIG 1. A, Serum ACE activity displayed as a function of ACE genotype. Significant differences ($P<.01$) between the genotypes were observed. B, Within-pair difference in MZ twins, DZ twins concordant for the ACE genotype, and DZ twins discordant for the ACE genotype. Similar small within-pair differences in MZ and concordant DZ twins were observed. Discordant DZ twins had a much greater within-pair difference ($P<.01$). This finding illustrates the overriding dominance of the ACE gene and its alleles in determining serum ACE activity levels.

tolic and diastolic blood pressure, and physical activity have a considerable effect on heart size, cardiac dimensions were corrected for these variables by stepwise multiple linear regression. For posterior wall thickness age, sex, and weight were the only variables entering the relationship, for septum thickness these were age, weight and systolic blood pressure.

Serum ACE activity levels and ACE genotypes are shown in Fig 1A. The linear regression of ACE activity over the number of D alleles attests to an significant allele-dose effect with a slope of 17. The variance was progressively greater in subjects with the ID and DD genotype than in subjects with the II genotype, resulting in a significant increase of the intrapair differences among MZ between genotypes. Variance differences were adjusted by log transformation of the difference from the genotype-specific mean. We next examined the twin pair difference in ACE activity. In Fig 1B is shown the difference in MZ twins, DZ twins concordant for the ACE genotype, and DZ twins discordant for the ACE genotype. MZ twins and concordant DZ twins showed the same, narrow within-pair difference in ACE activity. Discordant DZ twins on the other hand showed a 10-fold greater within-pair difference in ACE activity ($P<.01$). These data attest to the sole influence of ACE genotypes on the serum ACE activity level in these subjects. We performed linear regression analysis to examine correlations between serum ACE activity levels and posterior wall thickness, with or without correction for gender, body size, and physical activity. These correlations were: $r=.15$, $P<.05$ uncorrected and $r=.22$, $P<.01$ corrected.

In Fig 2A are shown the corrected posterior wall thickness values in the three ACE genotypes. Posterior wall thickness increases progressively with addition of the D allele ($P<.05$). In Fig 2B is shown the within-pair difference in MZ twins, concordant DZ twins, and discordant DZ twins. MZ twins showed a small degree of within-pair difference, while the within-pair difference in concordant and discordant DZ twins was significantly greater ($P<.05$). These data suggest that, in addition to ACE genotypes, other genes influence posterior wall thickness. Table 2 shows the genetic variance on ACE levels and on cardiac size. The additive genetic effects, shared, and non-shared environmental effects were all highly significant. In Table 3 are shown the demographic and clinical data on the subject population broken down in terms of AGT genotypes. No significant differences were found. The AGT levels increased numerically with addition of the T allele; however, the variability of the levels was too great to show significance.

Discussion

We documented the effect of genetic variance on echocardiographically determined cardiac dimensions and serum ACE activity levels. We verified that the ACE I/I genotype is associated with low serum ACE activity lev-

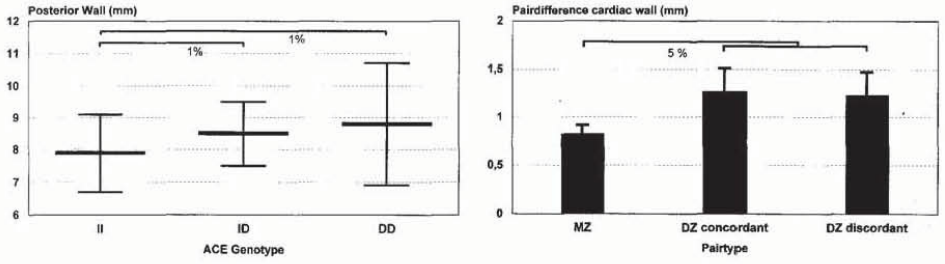


FIG 2. A, Corrected posterior wall thickness displayed as a function of ACE genotype. Significant differences ($P<.01$) between the genotypes were observed. (B, Within-pair difference in MZ twins, DZ twins concordant for the ACE genotype, and DZ twins discordant for the ACE genotype. A small within-pair difference was observed in MZ twins, while the concordant and discordant DZ twins had a much greater within-pair difference ($P<.01$). This finding suggests that posterior wall thickness is influenced not only by the ACE gene but also by other genes.

TABLE 2. Genetic Variance on ACE Levels and Cardiac Dimensions

Variables	A^2	C^2	E^2	χ^2	rMZ	rDZ	P
ACE	0.49	0.31	0.20	1.97/3	0.80	0.58	<.01
Posterior wall	0.26	0.20	0.54	3.82/3	0.48	0.26	<.01
Septum	0.37	0.24	0.39	3.61/3	0.64	0.37	<.01

A^2 indicates additive genetic effect; C^2, common or shared environment; and E^2, nonshared environmental effect.

els, the I/D genotype with intermediate levels, and the D/D genotype with high serum ACE activity levels. We were able to show a correlation between serum ACE activity levels and posterior wall thickness. We observed a small within-pair difference in serum ACE activity levels in MZ and concordant DZ twins, while in discordant DZ twins the difference was much greater. This observation gives strong support to the notion that the D allele exercises a strong, independent effect on serum ACE activity levels, and that the ACE gene alone is primarily responsible for ACE activity in normal individuals.

McKenzie et al[17] used a segregation and linkage analysis and found evidence for two quantitative-trait loci influencing ACE levels. Our findings are at variance with these observations and rather suggest that the ACE gene locus is primarily responsible for ACE levels. Morrison et al[18] studied African-Caribbean families from Jamaica, who may have differed genetically from our twin subjects. We tested our assumption with two different approaches. First, we corrected the ACE levels for the influence of the ACE gene by regression analysis and tested the residuals for remaining genetic effects. No significant additive or nonadditive genetic influences were discernible. Second, we compared the intrapair differences between MZ and concordant and discordant DZ twins. Were a second gene locus exerting an effect, the within-pair difference of concordant DZ twins would be expected to lie between those of MZ and DZ discordant twins. However, we observed no significant within-pair difference between MZ and concordant DZ twins. Instead, their values were almost identical. Twin methodology has substantial power for testing such hypotheses, as was shown in other studies.[18]

We found that the uncorrected posterior wall thickness was greater in subjects with the DD genotype than in subjects with the II or ID genotypes. Correcting the posterior wall thickness for gender, body size, and physical activity accentuated these differences. The within-pair difference was small in MZ twins, but similarly great in concordant and discordant DZ twins, attesting to the fact that posterior wall thickness is not merely a function of ACE genotypes but rather that other (perhaps many) genes are involved. Finally, our data are the first to show a correlation between serum ACE activity and any parameter of cardiac size. We have no immediate explanation on our failure to find a similar effect of the DD genotype on septal thickness. The effects we observed were small and our subjects were all normotensive. Possibly, the distribution of ACE in ventricular tissue is not uniform. ACE has been documented in the endocardium and coronary vasculature.[19] Danser et al[20] measured ACE activity in human left ventricular wall and found higher cardiac ACE levels in persons with the DD genotype. No comparisons of ACE levels in septum and posterior wall have been made; however, conceivably differing distributions of ACE could be responsible for differences in local growth effects.

TABLE 3. Demographic and Clinical Data in Groups With Different AGT Genotypes

Variables	MM	MT	TT	P
No.	18	34	17	
Age, y	33±14	32±14	35±13	NS
Sex, M/F	6/12	12/22	4/13	NS
Height, cm	167±8	168±9	170±9	NS
Weight, kg	65±13	66±13	69±12	NS
BMI, kg/m^2	23±4	23±4	23±4	NS
Systolic BP, mm Hg	123±17	122±15	123±14	NS
Diastolic BP, mm Hg	73±10	72±11	74±11	NS
HR, bpm	74±13	71±12	70±11	NS
ACE, U/L	43±18	42±22	41±23	NS
AGT, mg Ang I/mL	1.24±0.27	1.27±0.35	1.29±0.33	NS
Posterior wall, mm	8.8±2.2	8.6±1.5	8.4±1.3	NS
Septum, mm	8.8±1.7	8.8±1.7	8.6±1.3	NS

BMI indicates body mass index; BP, blood pressure; and HR, heart rate. Values are mean±SD.

Schunkert et al[5] studied a large random sample of men and women. With electrocardiographic criteria, they identified 141 women and 149 men with left ventricular hypertrophy. Among these subjects, an excess were homozygous for the D allele of the ACE gene. The association of the DD genotype with left ventricular hypertrophy was stronger in men than women, and was most prominent when blood pressure measurements were normal. They did not measure ACE levels in their subjects. Since their report, the ACE gene DD genotype has been associated with left ventricular hypertrophy by a group of Japanese investigators.[21] Furthermore, Marian et al[22] reported that the D allele was associated with hypertrophic cardiomyopathy and that the D allele was a risk for sudden cardiac death. They have extended these findings to show that the D allele is associated with the degree of hypertrophy in this disease.[6,23] They did not report the result of ACE measurements, and so the conclusion rests on the basis that ACE may be a genetic marker for left ventricular hypertrophy. Lindpaintner[1] subsequently commented that the diallelic polymorphism is only modestly informative and, since it resides in an intron, is extremely unlikely to represent a biologically relevant mutation.

Our results confirm the finding that the I/D polymorphism is associated with serum ACE activity.[2,18] Cambien et al[4] postulate the existence of an ACE Ss polymorphism, which has not yet been characterized at a molecular level. They suggest that the I/D polymorphism is a marker for the postulated functional variant ACE Ss and is associated with 28% to 44% of the interindividual variability of plasma ACE activity.[4] Our finding that serum ACE activity levels are correlated with posterior wall thickness, coupled with the relationship between the I/D genotypes and posterior wall thickness is evidence that the D allele is not merely a potential genetic marker but rather that plasma ACE activity is related to the development of left ventricular size, independent of blood pressure. Our findings conflict with the observations of Kupari et al,[24] who measured

left ventricular size and function but not serum ACE activity levels in relation to ACE polymorphisms in normal subjects. They studied 86 individuals, fewer than the number reported here. Furthermore, they did not apply the power of the twin model to test the hypothesis. Lindpaintner et al[25] also recently reported the absence of association or genetic linkage between ACE gene and left ventricular mass. They relied on echocardiographic data from the Framingham study and examined 759 sibling pairs in their linkage analysis. We have no immediate explanation for the discrepancy. The numbers of sibling pairs in their study is impressive. Echocardiography is a sensitive method of determining cardiac dimensions. They did not report on posterior wall thickness, which was the only ACE genotype-related parameter of cardiac size we identified. Echocardiography for the Framingham study was performed by numerous people over a period of years. The method does have errors and observer variability. There may have been a substantial within-pair age difference in the Framingham siblings. The twin model used is an extension of the sib-pair linkage approach. Our twin pairs were identical in age, which renders less variability. All echocardiograms were performed by the same experienced cardiologist in our study.

We cannot conclude for certain that the ACE DD genotype and increased ACE levels were responsible for increased posterior wall thickness in our population. The causative gene could be linked to the ACE gene locus and thereby show allelic association. Since the ACE locus had such a dominant effect on ACE levels, the correlation between ACE levels and posterior wall thickness does not necessarily strengthen the case for ACE. Nevertheless, there are several mechanisms whereby ACE could influence cardiac hypertrophy. For instance, increased ACE expression has been shown in the hearts of rats with pressure-overload–induced cardiac hypertrophy.[26] Ang I is converted to Ang II by ACE in the heart and elsewhere.[27-30] Ang II is associated with cell growth and can interact with several oncogenes such as c-*myc*, c-*fos*, and c-*jun*, which are known to be growth regulators.[31,32]

In addition to ACE, we also directed our attention to ATG and the ATG gene. We were only able to analyze ATG plasma concentrations from men and from women not ingesting oral contraceptives. We found lower ATG concentrations in subjects with the ACE DD genotype than in subjects with the ACE I allele. Conceivably, higher ACE levels may have led to an increase in AGT consumption. However, renin is considered to be most important in terms of generating Ang II.[33] We have recently been able to show in animal experiments that ACE gene expression and ACE activity in the vessel wall are a rate limiting step in Ang II generation.[34] We found no effect of AGT M/T genotypes on blood pressure or heart size. We were not able to document an effect of AGT M/T genotypes on AGT concentrations; however, the number of AGT samples in our study was limited to male twins only and may have been too small to test that hypothesis.

We conclude that the ACE gene locus is primarily responsible for ACE levels in our subjects and that the ACE DD genotype exerts an influence on cardiac posterior wall thickness independent of blood pressure. The correlation between ACE levels and posterior wall thickness suggests that ACE is indeed responsible. The AGT M/T polymorphism, in contrast, had no demonstrable effect on cardiac dimensions. These data support the notion that the ACE gene exerts an influence on cardiac size and development through the actions of ACE.

Acknowledgments

Andreas Busjahn is supported by a grant-in-aid from the Leopoldina Stiftung. Herbert Schuster is a recipient of a Hess Fellowship from the Deutsche Forschungsgemeinschaft. Friedrich C. Luft is supported by a grant-in-aid from the Bundesministerium für Bildung und Forschung.

References

1. Lindpaintner K. Genes, hypertension, and cardiac hypertrophy. *N Engl J Med.* 1994;330,1678-1679.
2. Tiret L, Rigat B, Visvikis S, Breda C, Corvol P, Cambien F, Soubrier F. Evidence, from combined segregation and linkage analysis, that a variant of the angiotensin I–converting enzyme (ACE) gene controls plasma ACE levels. *Am J Hum Genet.* 1992;51:197-205.
3. Costerousse O, Allegrini J, Lopez M, Alhenc-Gelas F. Angiotensin I–converting enzyme in human circulating mononuclear cells: genetic polymorphism of expression in T lymphocytes. *Biochem J.* 1993;290:33-40.
4. Cambien F, Costerousse O, Tiret L, Poirier O, Lecerf L, Gonzales MF, Evans A, Arveiler D, Cambou JP, Luc G, Rakotovao R, Ducimetiere P, Soubrier F, Alhenc-Gelas F. Plasma level and gene polymorphism of angiotensin-converting enzyme in relation to myocardial infarction. *Circulation.* 1994;90:669-676.
5. Schunkert H, Hense H-W, Holmer SR, Stender M, Perz S, Keil U, Lorell BH, Riegger GAJ. Association between and deletion polymorphism of the angiotensin-converting-enzyme gene and left ventricular hypertrophy. *N Engl J Med.* 1994;330:1634-1638.
6. Lechin M, Quinones MA, Omran A, Hill R, Yu Q-T, Rakowski H, Wigle D, Liew CC, Sole M, Roberts R, Marian AJ. Angiotensin-I converting enzyme genotypes and left ventricular hypertrophy in patients with hypertrophic cardiomyopathy. *Circulation.* 1995;92:1808-1812.
7. Jeunemaitre X, Soubrier F, Kotelevtsev Y, Lifton RP, Williams CS, Charru A, Hunt SC, Hopkins PN, Williams RR, Lalouel J-M, Corvol P. Molecular basis of human hypertension: role of angiotensinogen. *Cell.* 1992;71:169-180.
8. Bloem L, Manatunga AK, Tewksbury DA, Pratt JH. The serum angiotensinogen concentration and variants of the angiotensinogen gene in white and black children. *J Clin Invest.* 1995;95:948-953.
9. Sahn DJ, DeMaria A, Kisslo J, Weymen A. Recommendations regarding quantitation in M-mode echocardiography: results of a survey of echocardiographic measurements. *Circulation.* 1978;58:1072-1083.
10. Hammond HA, Jin L, Zhong Y, Caskey CT, Chakraborty R. Evaluation of 13 short tandem repeat loci for use in personal identification applications. *Am J Hum Genet.* 1994;55:175-189.
11. Rigat B, Hubert C, Alhenc Gelas F, Cambien F, Corvol P, Soubrier F. An insertion/deletion polymorphism in the angiotensin I–converting enzyme gene accounting for half the variance of serum enzyme levels. *J Clin Invest.* 1990;86:1343-1346.
12. Ward K, Hata A, Jeunemaitre X, Helin C, Nelson L, Namikawa C, Farrington PF, Ogasawara M, Suzumori K, Tomoda S, Berrebi S, Sasaki M, Corvol P, Lifton RP, Lalouel J-M. A molecular variant of angiotensinogen associated with preeclampsia. *Nat Genet.* 1994;4:59-61.
13. Benceteau B, Baudin B, Morgant C, Giboudeau J, Baumann FC. Automated kinetic assay of angiotensin-converting enzyme in serum. *Clin Chem.* 1986;32:884-886.
14. Bohlender J, Ménard J, Wagner J, Luft FC, Ganten D. Human renin-dependent hypertension in transgenic hAOGEN rats. *Hypertension.* 1996;27:535-540.
15. Jöreskog KG, Sörbom D. *LISREL 8.* Chicago, Ill: Scientific Software International Inc; 1993.
16. Neale MC, Cardon LR. *Methodology for Genetic Studies of Twins and Families.* Dordrecht, Netherlands: Kluwer Academic Publishers; 1992:496.
17. McKenzie CA, Julier C, Forrester T, McFarlane-Anderson N, Keavney B, Lathrop GM, Ratcliffe PJ, Farrall M. Segregation and linkage analysis of serum angiotensin I–converting enzyme levels: evidence for two quantitative-trait loci. *Am J Hum Genet.* 1995;57:1426-1435.
18. Morrison NA, Cheng Qi J, Tokita A, Kelly PJ, Crofts L, Nguyen TV, Sambrook PN, Eisman JA. Prediction of bone density from vitamin D receptor alleles. *Nature.* 1994;367:284-287.

19. Lindpaintner K, Ganten D. The cardiac renin-angiotensin system: an appraisal of present experimental and clinical evidence. *Circ Res.* 1991;68:905-921.
20. Danser AHJ, Schalekamp MADH, Bax WA, Maassen van den Brink A, Saxena PR, Riegger GAJ, Schunkert H. Angiotensin-converting enzyme in the human heart. *Circulation.* 1995;92:1387-1388.
21. Ohishi M, Rakugi H, Ogihara T. Association between a deletion polymorphism of the angiotensin converting enzyme gene and left ventricular hypertrophy. *N Engl J Med.* 1994;331:1097-1098.
22. Marian A, Yu Q, Workman R, Gottfried G, Roberts R. Angiotensin-converting enzyme polymorphism in hypertrophic cardiomyopathy and sudden death. *Lancet.* 1993;342:1085-1086.
23. Lechin M, Yu QT, Prather A, Hill R, Quinones M, Roberts R, Marian AJ. Angiotensin converting enzyme genotype DD is associated with increased left ventricular mass in patients with hypertrophic cardiomyopathy. *Circulation.* 1994;90(suppl I):I-174. Abstract.
24. Kupari M, Perola M, Koskinen P, Virolainen J, Karhunen PJ. Left ventricular size, mass, and function in relation to angiotensin-converting enzyme gene polymorphism in humans. *Am J Physiol.* 1994;267:H1107-H1111.
25. Lindpaintner K, Lee MinAe, Larson MG, Rao VS, Pfeffer MA, Ordovas JM, Schaefer EJ, Wilson AF, Wilson PWF, Vasan RS, Myers RH, Levy D. Absense of association or genetic linkage between the angiotensin-converting-enzyme gene and left ventricular mass. *N Engl J Med.* 1996;334:1023-1028.
26. Schunkert H, Jackson B, Tang SS, Schoen FJ, Smits JFM, Apstein CS, Lorell BH. Distribution and functional significance of cardiac angiotensin converting enzyme in hypertrophied rat hearts. *Circulation.* 1993;87:1328-1329.
27. Campbell DJ. The site of angiotensin production. *J Hypertens.* 1985;3:199-207.
28. Bünning P, Buneck W, Escher R, Schönherr E. Characteristics of angiotensin converting enzyme and its role in the metabolism of angiotensin I by endothelium. *J Cardiovasc Pharmacol.* 1986;8(suppl 10):S52-S57.
29. Soubrier F, Hubert C, Testut P, Nadaud S, Alhenc-Gelas F, Corvol P. Molecular biology of the angiotensin I converting enzyme, I: biochemistry and structure of the gene. *J Hypertens.* 1993;11:471-476.
30. Soubrier F, Wei L, Hubert C, Clauser E, Alhenc-Gelas F, Corvol P. Molecular biology of the angiotensin I converting enzyme, II: structure-function: gene polymorphism and clinical implications. *J Hypertens.* 1993;11:599-604.
31. Naftilan AJ, Pratt RE, Dzau VJ. Induction of platelet-derived growth factor A-chain and c-myc gene expression by angiotensin II in cultured rat vascular smooth muscle cells. *J Clin Invest.* 1989;83:1419-1424.
32. Jackson TR, Blair LA, Marshall J, Goedert M, Hanley MR. The mas oncogene encodes an angiotensin receptor. *Nature.* 1988;335:437-440.
33. Kato H, Iwai N, Inui H, Kimoto K, Uchiyama Y, Inagami T. Regulation of vascular angiotensin release. *Hypertension.* 1993;21:446-454.
34. Müller DN, Bohlender J, Hilgers KF, Dragun D, Costerousse O, Ménard J, Luft FC. Vascular expression of the angiotensin converting enzyme gene regulates the rate of local angiotensin II formation. *Hypertension.* 1997;29(part 1):98-104.

Heritability Analysis of Lipids and Three Gene Loci in Twins Link the Macrophage Scavenger Receptor to HDL Cholesterol Concentrations

Hans Knoblauch, Andreas Busjahn, Sylvia Münter, Zsuzsanna Nagy, Hans-Dieter Faulhaber, Herbert Schuster, Friedrich C. Luft

Abstract We studied 100 healthy monozygotic and 72 dizygotic twin pairs (mean age, 34±14 years) to test for genetic influences on blood lipids and to examine relevant gene loci. Total cholesterol (TC), LDL cholesterol (LDL-C), HDL cholesterol (HDL-C), and triglyceride (TG) levels were determined after a 12-hour fast. Zygosity was determined with the use of microsatellite markers. Heritability estimates were conducted by using the lisrel 8 program; a sib-pair analysis was conducted by using the sibpal program. Linear regression analyses were carried out between identical-by-descent status and squared within-pair differences of TC, LDL-C, HDL-C, and TG values. Heritability estimates of the lipid serum concentrations ranged from.58 to.66. A significant linkage relationship was found for HDL-C (P=.008) and TGs (P=.05) with D8S261 on chromosome 8p. However, no linkage was found between any of the lipid variables and the lipoprotein lipase gene locus (LPL GZ14/15 and D8S282). Because D8S261 is located approximately halfway between the LPL and macrophage scavenger receptor genes, we examined the nearby markers D8S549 and D8S1731. Linkage was found for HDL-C and D8S549 (P=.001) and for HDL-C and D8S1731 (P=.04). On the other hand, we found no linkage between the LDL receptor gene locus and LDL-C serum concentrations nor between the LPL gene locus and the various other lipid fractions. Our data suggest a significant influence of the macrophage scavenger receptor gene locus on HDL-C and weak influence on TG levels. We suggest that inherited variability in the macrophage scavenger receptor gene has an influence on serum lipid concentrations. (***Arterioscler Thromb Vasc Biol.* 1997;17:2054-2060.**)

Key Words • genetics • HDL cholesterol • lipoprotein lipase • LDL cholesterol • macrophage scavenger receptor

Coronary heart disease, particularly at a young age, is largely influenced by genetic variance.[1,2] Because serum TC, LDL-C, and TG levels are directly correlated with cardiovascular risk and HDL-C is inversely correlated with cardiovascular risk,[3,4] the influence of genetic variance on these serum lipids is of great interest. However, the heritability data are not entirely clear and are in part conflicting.[5] MZ and DZ twins provide a classic model with which to determine estimates of the influence of heredity and environment on various traits, including the risk for CHD and detrimental serum lipid levels.[6-8] Twin studies also provide an opportunity to examine possible linkage between genetic loci and phenotypic traits in terms of a modified sibling-pair analysis.[9] Although LDLR gene mutations have dramatic effects on circulating LDL-C levels in persons with familial hypercholesterolemia,[10] the influence of the LDLR gene on LDL-C concentrations in the general population is less clear.[11-13] Furthermore, data on the influence of the LPL gene locus on lipoprotein and TG levels do not uniformly agree.[14-18] This state of affairs may be related to the use of neutral polymorphisms in some studies[14,15] compared with functional polymorphisms in others.[19-21] We studied 100 MZ and 72 DZ pairs of healthy twins to determine the influence of heredity and environment on TC, LDL-C, HDL-C, and TG levels. We first tested the loci for the LDLR and LPL genes. We chose the LPL gene because of the aforementioned association studies. When we found no linkage at either locus, we directed our attention to the nearby macrophage scavenger receptor gene locus for influences on lipoprotein concentrations.

Received January 14, 1997; revision accepted May 15, 1997.

From the Franz Volhard Clinic and Max Delbrück Center for Molecular Medicine, Virchow Klinikum, Humboldt University of Berlin, Berlin, Germany.

Correspondence to Friedrich C. Luft, MD, Franz Volhard Clinic, Wiltbergstrasse 50, 13122 Berlin, Germany.

E-mail fcluft@mdc-berlin.de

Methods

General Procedures

We recruited 172 pairs of twins (100 MZ) and (72 DZ) by advertisement to participate in studies involving blood pressure regulation and cardiovascular phenotypes.[22,23] The subjects were all healthy, normotensive whites of German ancestry from various parts of Germany. The protocol was approved by the University's committee on the protection of human subjects, and written, informed consent was obtained from all participants. Women who were using oral contraceptives or estrogen preparations, women >50 years old, and individuals of both sexes who were ingesting lipid-influencing medications were excluded from this analysis. Persons with histories of familial lipid disorders were also excluded. Blood was obtained from all twins after a 12-hour fast. TC, HDL-C, and TG levels were determined by automated methods.[24] LDL-C concentrations were calculated by the Friedewald equation.[25] Blood was also obtained for determination of zygosity and other molecular genetic studies.

Molecular Genetic Methods

Zygosity was verified by the use of five PCR-amplified microsatellite markers as described in detail elsewhere.[26] In brief, we used five highly polymorphic short-tandem-repeat loci

Selected Abbreviations and Acronyms

CHD = coronary heart disease
DZ = dizygotic
IBD = identical by descent
LDLR = LDL receptor
LPL = lipoprotein lipase
MZ = monozygotic
PCR = polymerase chain reaction
TC = total cholesterol
TG = triglyceride

that were coamplified by PCR with the use of fluorescence-labeled primers. Four markers were multiplexed simultaneously, while the fifth was run separately. Thirty-six samples were electrophoresed and detected simultaneously by laser. The PCR products were sized by automated fragment analysis. We modified our reaction slightly to include six additional markers, namely, D8S261 and D8S549, D8S1731 and LPL GZ 14/15, and D8S282 and D19S394, which are in close proximity to the macrophage scavenger receptor, LPL, and LDLR genes, respectively.[27] The PCR reactions were performed in a final volume of 15 μL containing dNTPs (200 mmol/L), primers (5 pmol), PCR reaction buffer (supplied by the manufacturer), $MgCl_2$ (1.5 mmol/L), and AmpliTaq gold (0.65 U). The annealing temperatures were 58°C for 56°C for D19S394 and D8S261, 52°C for D8S549, 44°C for D8S1731, 56°C for D8S282, and 56°C for LPL GZ 14/15.

Twin Analysis Methods

Linkage analyses were carried out using the sibpal program of the Statistical Analysis for Genetic Epidemiology (SAGE) package.[28] The underlying basis for the sib-pair linkage approach is to compare the quantitative variation in a trait between siblings as a function of the number of marker alleles that they share IBD. Because parental genotypes were not available, we estimated the number of IBD alleles on the basis of allele frequencies from each twin in each pair separately. Estimates were calculated by the sibpal program. The underlying trait can follow either mendelian or nonmendelian modes of inheritance. We assessed linkage for continuous traits, such as LDL-C, HDL-C and TGs, against candidate gene loci as described elsewhere.[29] Because we used a candidate gene approach, we accepted $P<.05$ to test for significance.

To test whether or not our observations were the result of chance alone, we performed a simulation analysis in which we examined pair differences with randomly allocated IBD 100 times. Our simulation analysis confirmed that the probability of a false-positive result was estimated correctly from the regression analysis. The average probability of false-positive results at the.05 level was.046. The probability of a false-positive result below the obtained probability value was <.001.

Statistical analysis was conducted with the spss program. To test for differences in mean levels for any given variable, *t* tests for independent groups were used. Parameters of the quantitative genetic models were estimated by path analysis techniques using the lisrel 8 program developed by Jöreskog and Sörbom.[30] Analogous to that obtained by regression analysis, the variability of any given phenotype *(P)* within a population can be partitioned into genetic influences *(A)*, environmental influences shared by twins within the same family *(C)*, and random environmental influences *(E)*: $P=aA+cC+eE$, with the coefficients a, c, and e as the estimated relative influence. For MZ and DZ twins, the covariance of their phenotype is given by $r_{MZ}=a^2+c^2+e^2$ and $r_{DZ}=0.5a^2+c^2+e^2$, respectively. Path analysis in twin studies can estimate additive and nonadditive (dominance) components of genetic variability (estimated as h^2 and d^2, respectively) as well as two environmental influences, shared (c^2) and unshared (e^2).[31] These values estimate the relative amount of the variable's influence on interindividual differences to a sum of 1. Genetic as well as environmental effects were estimated by a best-fit model selected by the χ^2 test. The lisrel 8 output also provides estimates of the goodness-of-fit index, the adjusted goodness-of-fit index, and the Akaike information criterion. Because these estimates concurred with those derived by χ^2 analysis, we have elected to not present them here.

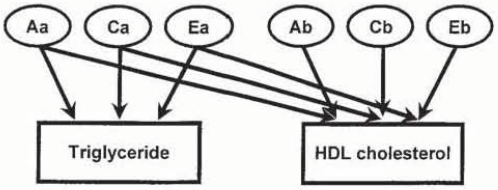


FIG 1. Bivariate path analysis model. *Aa* is a set of genes influencing both HDL-C and TGs; *Ab* is a set of genes specific for TG level; *C* and *E* are environmental influences within and between families.

The hypothesis that different genes influence lipid fractions can be examined by a bivariate path analysis.[32] The basic structure of the model, which assumes only additive genetic effects, is displayed in Fig 1. This model includes two sets of genes, one set that influences both phenotypes (eg, TGs and HDL-C; A_a), and the second set that influences the second phenotype only (A_b), two sets of shared (C_a and C_b), and unshared (E_a and E_b) environmental factors. For the first phenotype the total genetic influence is estimated; for the second phenotype the genetic variance is divided into common and specific factors.

Results

Table 1 shows the demographic and lipid-related variables for the 100 pairs of MZ twins and the 72 pairs of DZ twins. The number of females represented was twice as great as the number of males. The subjects were generally young adults of normal height, weight, and body mass index. TC, HDL-C, LDL-C, and TG values were all within normal limits.

Table 2 shows the results of the heritability analysis. A major genetic effect was demonstrated for all lipid parameters, although strong environmental effects were also demonstrated. A slight albeit significant shared environmental effect was also observed for HDL-C. Fig 2 shows the genetic and environmental effects shared by two phenotypes, namely, HDL-C with TGs, LDL-C with TGs, and HDL-C with LDL-C in a combined analysis.

TABLE 1. Clinical Data and Serum Lipid Values

Variable	Total	MZ	DZ
No. of pairs	172	100	72
Age, y	33±14	33±15	34±12
Sex, M/F	103:241	52:148	51:93
Height, cm	169±9	168±9	171±8
Weight, kg	68±13	66±12	70±14
Body mass index, kg/m^2	23±4	23±4	23±4
Systolic blood pressure, mm Hg	123±15	125±16	120±11
Diastolic blood pressure, mm Hg	73±11	73±11	73±9
TC, mg/dL	189±39	190±39	187±39
HDL-C, mg/dL	53±15	54±15	52±15
LDL-C, mg/dL	117±33	117±34	115±32
TGs, mg/dL	95±64	95±65	95±62

All values except those for sex are mean ± SD.

TABLE 2. Genetic and Environmental Effects on Serum Lipid Values

	Genetic Effect	Environmental Effect		χ^2/df	r_{MZ}	r_{DZ}
		Shared	Nonshared			
TC	.58		.42	6.1/4	.58	.28
HDL-C	.61	.07	.32	3.9/3	.69	.35
LDL-C	.59		.41	2.7/4	.60	.11
TGs	.66		.34	11.9/4	.68	.28

Only significant ($P<.05$) relationships are shown.

The *y* axis shows the total genetic and environmental influences on the given lipid variable pairs as a percentage. HDL-C and TGs shared common family environmental and common genetic effects, which comprised ≈20% of the total variance. LDL-C and TGs shared only a small amount (10%) of common genetic effects. HDL-C and LDL-C shared neither genetic nor environmental influences.

Table 3 shows the probability values for the regression analysis performed to examine the relationship between IBD versus within-pair difference at the three loci in question. A significant linkage relationship was found for HDL-C ($P=.008$) and TGs ($P=.05$) with D8S261. Similarly, linkage was found for HDL-C and D8S549 ($P=.001$) and D8S1731 ($P=.04$). On the other hand, no linkage was found between any of the lipid variables and the LPL gene loci (LPL GZ14/15 and D8S282) or the LDLR gene locus (D19S394). Fig 3 is a map of the area in question on chromosome 8p. The macrophage scavenger receptor gene locus and the LPL gene locus are ≈9 cM apart from each another. The marker locations that we tested are also shown on the figure.[27]

Discussion

The important findings in this study are that TC, HDL-C, LDL-C, and TGs are all equally influenced by both genetic and environmental influences. A similar shared-environmental effect was observed between HDL-C and TG values. Lesser concordance of genetic effects was observed when HDL-C versus TG values and LDL-C versus TG values were compared. Environmental and genetic effects between HDL-C and LDL-C appeared to be entirely separate. When we examined the three marker gene loci, the LDLR gene locus was not linked to any of them. On the other hand, the marker D8S261 was linked to TG concentrations. An effect of this locus on HDL-C concentrations was also observed. D8S261 lies on chromosome 8p in proximity to loci for the LPL gene and the macrophage scavenger receptor gene. The chromosome map indicates that the genes are ≈9 cM apart. We next examined a microsatellite marker within the LPL gene (LPL GZ14/15) as well as another marker (D8S282) very nearby. Evaluation of these markers suggested no linkage between serum lipid concentrations and the LPL gene locus. However, when we examined markers closer to the macrophage scavenger receptor, linkage was found for HDL-C and D8S549 and HDL-C and D8S1731. To our knowledge, these results are the first demonstration of linkage between any serum lipid concentration and the macrophage scavenger receptor gene locus. Of course, we cannot be certain that the macrophage scavenger receptor gene alone is responsible for these findings, since another unknown locus influencing lipoprotein metabolism, distinct from the macrophage scavenger receptor gene but within 5 cM, could also be responsible.

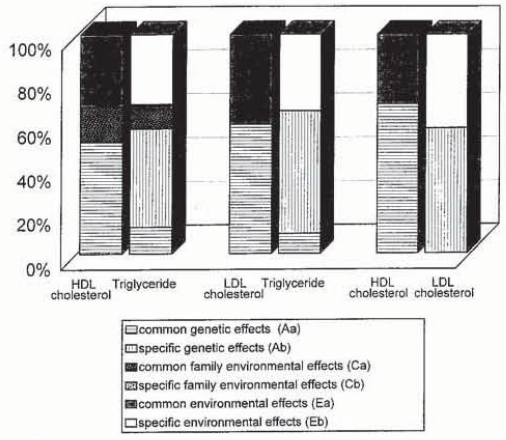


FIG 2. Effect of genetic and environmental effects shared by two phenotypes. The *y* axis shows the total genetic and environmental influence on the given lipid variable pairs. HDL-C and TGs shared common family environmental effects and, to a lesser degree, common genetic effects. LDL-C and TGs shared only a small amount of genetic effects. HDL-C and LDL-C shared neither genetic nor environmental influences.

Numerous studies have examined genetic and environmental influences on serum lipid levels in twins.[5,6,33-38] The most comprehensive study in terms of defining the effects of genetics and environment was the Swedish Adoption/Twin Study of Aging (SATSA).[6] This remarkable study involved 302 pairs of twins, of which 146 pairs had been reared apart. Furthermore, the age range of the twins was sufficient to allow insight into age-related effects. The heritability of lipid serum levels ranged from.28 to.78 in that study. The environment of rearing (shared) had a substantial impact on the levels of TC but not on those of HDL-C or TGs. The influence of heredity, particularly for TGs, decreased with age. Our estimates of heritability, as well as shared and unshared environmental effects, is in basic agreement with the SATSA results for young adult twins.[6] We were unable to test age-related hypotheses because of the narrow age range of our subjects. However, our primary hypotheses were not related to heritability estimates, environmental effects, or age-related effects but rather to a possible linkage between serum lipid levels and two gene loci, namely, the LDLR gene and the LPL gene loci.

We were unable to find any linkage between the LDLR gene locus and LDL-C concentrations in the twins. Our approach may be criticized because we did not use flanking markers on either side of the LDLR gene locus, and recombinations admittedly may have occurred. However, we think that this possibility is unlikely because the microsatellite on chromosome 19 resides within 250 kb of the LDLR gene. Furthermore, Haddad et al[39] used the same marker in a study of patients with familial hypercholesterolemia and found no recombinations. Earlier studies found associations between polymorphisms of the LDLR gene and LDL-C

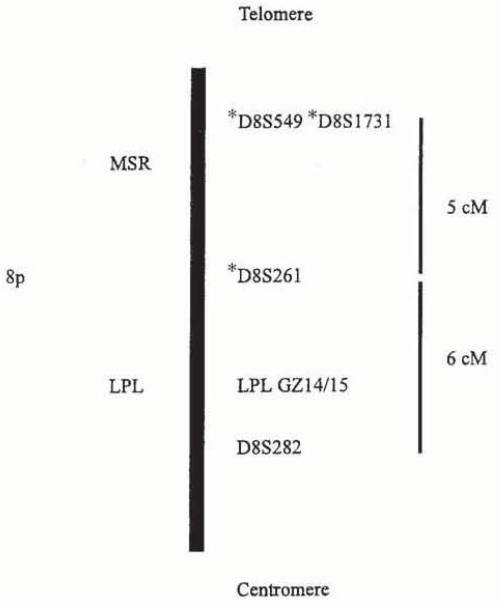
TABLE 3. Linkage Between a Given Variable and Markers at the Macrophage Scavenger Receptor (MSR), LPL, and LDLR Gene Loci

Phenotype	Locus	Marker	*df*	*t*	*P*	Intercept	Slope
TC	MSR	D8S549	46	2.20	.98	−636.79	4013.09
		D8S1731	48	0.58	.72	1226.62	835.53
		D8S261	58	−0.50	.31	1639.54	−591.31
	LPL	LPL GZ 14/15	51	−0.45	.33	2082.75	−690.79
		D8S282	58	−0.70	.24	1745.49	−770.86
	LDLR	D19S394	58	−0.65	.26	1592.37	−564.52
LDL-C	MSR	D8S549	46	1.58	.94	124.78	2003.62
		D8S1731	48	0.54	.70	1000.13	580.75
		D8S261	58	−0.57	.29	1469.52	−596.02
	LPL	LPL GZ 14/15	51	−1.07	.14	2058.37	−1257.03
		D8S282	58	−1.21	.11	1789.99	−1174.87
	LDLR	D19S394	58	−0.42	.34	1317.09	−327.21
HDL-C	MSR	D8S549	46	−3.15	.001	970.46	−970.46
		D8S1731	48	−1.77	.04	308.81	−201.87
		D8S261	58	−2.48	.008	509.52	−509.52
	LPL	LPL GZ 14/15	51	−1.34	.093	370.41	−281.73
		D8S282	58	−1.10	.14	361.41	−221.44
	LDLR	D19S394	58	−0.98	.16	319.70	−156.69
TGs	MSR	D8S549	46	0.85	.80	1660.82	7104.58
		D8S1731	48	−0.79	.22	7607.06	−4202.55
		D8S261	58	−1.66	.05	8588.68	−8363.63
	LPL	LPL GZ-14/15	51	−0.57	.29	6397.95	−3219.60
		D8S282	58	−0.63	.27	5747.30	−3018.16
	LDLR	D19S394	58	−0.60	.28	5365.06	−2260.79

serum concentrations. Pedersen and Berg[40] found that persons homozygous for the absence of the *Pvu* II restriction site at the LDLR gene locus had a higher chance of being in the uppermost quartile of TC levels. We were able to confirm this association in a normocholesterolemic German population.[41] Humphries et al[42] examined four restriction fragment length polymorphisms at the LDLR gene locus in an Italian population. They confirmed the *Pvu* II polymorphism association and also observed an association between the LDL-C–lowering *P2* allele and increased survival for those >65 years. We studied the *Pvu* II polymorphism with a novel, anchored PCR in three populations (Iceland, Scotland, and England).[43] When the two groups from the United Kingdom were combined, a significant association between the *T/T* genotype, compared with other genotypes, and lower TC and TG values was identified. Ahn et al[44] studied the *Ava* II and the *Nco* I polymorphism in the LDLR genes of Hispanic and non-Hispanic Americans. Both polymorphisms revealed an effect on TC and LDL-C; however, the effects were confined to women only.

We believe that the number of twins in our study was sufficient to find linkage between the LDLR gene locus and LDL-C serum concentrations, had it been present. Indeed, we did find linkage between markers in proximity to the macrophage scavenger receptor gene locus and HDL-C and, though not as strong, to serum TGs. Greenberg[45] has provided a careful discussion to explain the apparent "discrepancies" in such findings. He pointed out the difference between so-called susceptibility gene loci, which are neither necessary nor sufficient to cause disease, and those loci that are necessary but may not be sufficient for disease expression. Susceptibility gene loci increase risk and may involve the existence of multiple interacting genes (epistasis) or a disease locus in linkage disequilibrium with the marker locus. Greenberg then used a computer simulation model in which the hypothetical allele increased the risk of disease expression by a factor of 10. Nevertheless, even with 30 nuclear families, each with two affected members, the chances of finding linkage were extremely low. Greenberg then expanded his argument by indicating that linkage analysis on risk factor data may not yield additional information about linkage in the usual sense but may help distinguish between different hypotheses to explain the association.

The role of TG concentrations in the development of CHD and the value of its measurement in predicting disease risk remain controversial.[46] TG is often not a significant predictor of CHD in multivariate statistical models because of the large variation in TG measurements and the strong inverse relation between HDL-C and TG levels.[47] LPL plays a role in determining the plasma lipid profile, since it is the rate-limiting enzyme in the clearance of TG-rich lipoproteins from the circulation.[48] This enzyme also influences apolipoprotein and phospholipid exchange between VLDL-C and HDL-C. LPL thereby affects inter-HDL-C conversions and LDL-C generation derived from VLDL clearance.[49] Mutations in the LPL gene and their influence on lipid levels, particularly TGs and HDL-C, have generated major interest.[14-21] Nevertheless, Heliö et al[17] were unable to find evidence for linkage between familial hypertriglyceridemia and the LPL gene. We were also unable to link the LPL gene locus with serum TG concentrations in these healthy twin subjects. We used one marker that lies within the gene and another very close to it. These markers should have been sufficiently informative to demonstrate linkage; however, it is possible that our numbers were not sufficiently large for this purpose. The



Chromosomal map of chromosome 8p.
MSR(Macrophage scavenger receptor gene)
LPL (Lipoprotein lipase gene).

FIG 3. Chromosome map of markers in the vicinity of the LPL gene and the macrophage scavenger receptor (MSR) gene on chromosome 8p.[27] Those markers in which linkage to HDL and/or TGs was found are indicated by an asterisk.

LPL gene resides on chromosome 8p, ≈9 cM from the macrophage scavenger receptor gene.

We found much more impressive results when we examined markers closer to the macrophage scavenger receptor gene locus. Interestingly, the linkage results between HDL-C and these markers were much more robust than those with TGs. These results are consistent with studies that have found associations between polymorphisms in the LPL gene and TG levels, HDL-C levels, and CHD.[14-21] For instance, persons heterozygous for LPL deficiency are known to have higher TG levels, lower HDL-C concentrations, and higher systolic blood pressures than LPL-normal individuals.[50,51] The latter interaction is of interest because of recent observations by Pimstone et al,[52] who presented evidence that mutations in the LPL gene may be a cause of low HDL-C levels in some individuals heterozygous for familial hypercholesterolemia. However, the aforementioned association studies could also be interpreted to indicate that polymorphisms in the LPL gene were in disequilibrium with a mutation in a nearby gene, namely, that for the macrophage scavenger receptor gene. We intend to apply multiplex sequencing techniques to both the LPL and the macrophage scavenger receptor genes in DZ twins to further examine these important issues.

Macrophage scavenger receptors are implicated in the pathological deposition of cholesterol (modified LDL-C) in macrophages during atherogenesis, resulting in foam cell formation.[53] Macrophage scavenger receptors bind a wide range of ligands, including TG-rich lipoproteins and even bacterial pathogens.[54] Targeted disruption of the macrophage scavenger receptor-*A* gene in mice resulted in reductions in the size of atherosclerotic lesions in animals deficient in apolipoprotein E.[55] The macrophages from these mice showed a marked decrease in modified LDL-C uptake in vitro, but in vivo modified-LDL clearance was not affected. We cannot explain the linkage of the macrophage scavenger receptor gene locus in DZ twins. However, the interrelationships between the various lipid fractions and the apparent alternative mechanisms of elimination that have not yet been elucidated lead us to speculate that variations in the macrophage scavenger receptor gene have an influence on HDL-C concentrations and therefore on the risk for atherosclerosis. We realize that this hypothesis remains speculative until functionally significant mutations in the gene have been identified. Finally, we cannot exclude the possibility that another neighboring gene is responsible.

In summary, we examined healthy MZ and DZ twins to test for linkage between the LDLR gene locus, LPL gene locus, and the macrophage scavenger receptor gene locus and serum lipid concentrations. We found evidence for linkage between the macrophage scavenger receptor gene locus and serum HDL-C values, as well as a weaker one to TG concentrations, but could find no linkage between the LDLR gene locus and serum LDL-C concentrations or between the LPL gene locus and the various lipid fractions. The latter observation in no way detracts from the results of earlier association studies but may instead be explained by the difference in susceptibility gene loci and those loci necessary for disease expression. We suggest that the macrophage scavenger receptor gene locus should receive increased attention in terms of atherosclerotic risk.

Acknowledgments

This study was supported by grants-in-aid from the Leopoldina Stiftung (to A.B.), the Deutsche Forschungsgemeinschaft (to H.S.), and the Bundesministerium für Bildung und Forschung (F.C.L.), and the Danone Corporation.

References

1. Goldstein JL, Schrott HG, Hazzard WR, Bierman EL, Motulsky AG. Hyperlipidemia in coronary heart disease, II: genetic analysis of lipid levels in 176 families and delineation of a new inherited disorder, combined hyperlipidemia. *J Clin Invest*. 1973;52: 1544-1568.
2. Marenberg ME, Risch N, Berkman LF, Floderus B, de Faire U. Genetic susceptibility to death from coronary heart disease in a study of twins. *N Engl J Med*. 1994;330:1041-1046.
3. Kannel WB, Castelli WP, Gordon T, McNamara PM. Serum cholesterol, lipoproteins, and the risk of coronary heart disease. *Ann Intern Med*. 1971;74:1-12.
4. Assmann G, Schulte H. Relation of high density lipoprotein cholesterol and triglycerides to incidence of atherosclerotic coronary artery disease: the PROCAM experience. *Am J Cardiol*. 1992;70: 733-737.
5. Breslow JL. Genetics of lipoprotein disorders. *Circulation*. 1993; 87(suppl III):III-16-III-21.
6. Heller DA, de Faire U, Pedersen NL, Dahlén G, McClearn GE. Genetic and environmental influences on serum lipid levels in twins. *N Engl J Med*. 1993;328:1150-1156.

7. Feinleib M, Garrison RJ, Fabsitz R, Christian JC, Itvubec Z, Bovhani NO, Uannel WB, Rosenman R, Swartz JT, Wagner JO. The NHLBI twin study of cardiovascular disease risk factors: methodology and summary of results. *Am J Epidemiol*. 1977;106:284-295.
8. Whitefield JB, Martin NG. Plasma lipids in twins: environmental and genetic influences. *Atherosclerosis*. 1983;48:265-277.
9. Morrison NA, Qi JC, Tokita A, Kelly PJ, Crofts L, Nguyen TV, Sambrook PN, Eisman JA. Prediction of bone density from vitamin D receptor alleles. *Nature*. 1994;367:284-287.
10. Brown MS, Goldstein JL. A receptor-mediated pathway for cholesterol homeostasis. *Science*. 1986;232:34-47.
11. Maartmann-Moe K, Berg-Johnsen P. Genetics of the low density lipoprotein receptor, I: low density lipoprotein receptor activity in cultured fibroblasts from subjects with or without familial hypercholesterolemia. *Clin Genet*. 1981;20:90-103.
12. Magnus P, Maartmann-Moe K, Golden W, Nance WE, Berg K. Genetics of the low density lipoprotein receptor, II: genetic control of variation in cell membrane low density lipoprotein receptor activity in cultured fibroblasts. *Clin Genet*. 1981;20:104-112.
13. Maartmann-Moe K, Magnus P, Golden W, Berg K. Genetics of the low density lipoprotein receptor, III: evidence for multiple normal alleles at the low density lipoprotein receptor locus. *Clin Genet*. 1981;20:113-129.
14. Jemaa R, Fumeron F, Poirier O, Lecerf L, Evans A, Arveiler D, Luc G, Cambou J-P, Bard J-M, Fruchart J-C, Apfelbaum M, Cambien F, Tiret L. Lipoprotein lipase gene polymorphisms: associations with myocardial infarction and lipoprotein levels, the ECTIM study. *J Lipid Res*. 1995;36:2141-2146.
15. Gerdes C, Gerdes LU, Hansen PS, Faergeman O. Polymorphisms in the lipoprotein lipase gene and their associations with plasma lipid concentrations in 40-year-old Danish men. *Circulation*. 1995; 92:1765-1769.
16. Mattu RK, Needham EWA, Morgan R, Rees A, Hackshaw AK, Stocks J, Elwood PC, Galton DJ. DNA variants at the LPL gene locus associate with angiographically defined severity of atherosclerosis and serum lipoprotein levels in a Welsh population. *Arterioscler Thromb Vasc Biol*. 1994;14:1090-1097.
17. Heliö T, Palotie A, Sane T, Tikkanen MJ, Kontula K. No evidence for linkage between familial hypertriglyceridemia and apolipoprotein B, apolipoprotein C-III or lipoprotein lipase genes. *Hum Genet*. 1994;94:271-278.
18. Peacock RE, Hansten A, Nilsson-Ehle P, Humphries SE. Associations between lipoprotein lipase gene polymorphisms and plasma correlations of lipids, lipoproteins and lipase activities in young myocardial infarction survivors and age-matched healthy individuals from Sweden. *Atherosclerosis*. 1992;97:171-185.
19. Mailly F, Tugrul Y, Reymer PWA, Bruin T, Seed M, Groenemeyer BF, Asplund-Carlson A, Vallance D, Winder AF, Miller GJ, Kastelein JJP, Hamsten A, Olivecrona G, Humphries SE, Talmud PJ. A common variant in the gene for lipoprotein lipase (Asp9→Asn): functional implications and prevalence in normal and hyperlipidemic subjects. *Arterioscler Thromb Vasc Biol*. 1995: 15:468-478.
20. Reymer PWA, Gagne E, Groenemeyer BE, Zhang H, Forsyth I, Jansen H, Seidell JC, Kromhout D, Lie KE, Kastelein J, Hayden MR. A lipoprotein lipase mutation (Asn291Ser) is associated with reduced HDL cholesterol levels in premature atherosclerosis. *Nat Genet*. 1995;10:28-33.
21. Jukema JW, van Boven AJ, Groenemeijer B, Zwinderman AH, Reiber JHC, Bruschke AVG, Henneman JA, Molhoek GP, Bruin T, Jansen H, Gagne E, Hayden MR, Kastelein JJP, on behalf of the REGRESS study group. The Asp9 Asn mutation in the lipoprotein lipase gene is associated with increased progression of coronary atherosclerosis. *Circulation*. 1996;94:1913-1918.
22. Busjahn A, Faulhaber H-D, Viken RJ, Rose RJ, Luft FC. Genetic influences on blood pressure with the cold pressor test: a twin study. *J Hypertens*. 1996;14:1195-1199.
23. Busjahn A, Knoblauch J, Knoblauch M, Schuster H, Bohlender J, Faulhaber H-D, Luft FC. Angiotensin converting enzyme and angiotensinogen gene polymorphisms, plasma levels, and left ventricular size: a twin study. *Hypertension*. 1997;29:165–170.
24. Allain CC, Poon IS, Chan CSG, Richmond W, Fu PC. Enzymatic determination of total serum cholesterol. *Clin Chem*. 1974;20: 470-475.
25. Friedewald WT, Levy RI, Fredrickson DS. Estimation of plasma low-density lipoprotein cholesterol concentration, without use of the preparative ultracentrifuge. *Clin Chem*. 1972;18:499-501.
26. Becker A, Busjahn A, Faulhaber H-D, Bähring S, Schuster H, Luft FC. Automated zygosity determination with microsatellites. *J Reprod Med*. 1997;42:260–266.
27. Dib C, Faure S, Fizames C, Samson D, Drouot N, Vignal A, Millasseau P, Marc S, Hazan J, Seboun E, Lathrop M, Gyapay G, Morissette J, Weissenbach J. A comprehensive genetic map of the human genome based on 5,264 microsatellites. *Nature*. 1996;380: 152-154.
28. *S.A.G.E.: Statistical Analysis for Genetic Epidemiology, release 2.2*. Cleveland, Ohio: Department of Epidemiology and Biostatistics, Case-Western Reserve University; 1994.
29. Wu D, Bu X, Warden CH, Shen DDC, Jeng C-Y, Sheu WHH, Fuh MMT, Katsuya T, Dzau VJ, Reaven GM, Lusis AJ, Rotter JI, Chen Y-D I. Quantitative trait locus mapping of human blood pressure to a genetic region at or near the lipoprotein lipase gene locus on chromosome 8p22. *J Clin Invest*. 1996;97:2111-2118.
30. Jöreskog KG, Sörbom D. *lisrel 8*. Chicago, Ill: Scientific Software International Inc; 1993.
31. Neale MC, Cardon LR. *Methodology for Genetic Studies of Twins and Families*. Dordrecht, Netherlands: Kluwer Academic Publishers; 1992.
32. Neale MC, Cardon LR. *Methodology for Genetic Studies of Twins and Families*. Dordrecht, Netherlands: Kluwer Academic Publishers; 1992:496.
33. Stistonen P, Ehnholm C. On the heritability of serum high density lipoprotein in twins. *Am J Hum Genet*. 1980;32:1-7.
34. Hunt SC, Hassstedt SJ, Kuida H, Stults BM, Hopkins PN, Williams RR. Genetic heritability and common environmental components of resting and stressed blood pressures, lipids, and body mass index in Utah pedigrees and twins. *Am J Epidemiol*. 1989;129:625-638.
35. Duccini GM, Colletto D, Krieger H, Magalhães JR. Estimates of the genetical and environmental determinants of serum lipid and lipoprotein concentrations in Brazilian twins. *Hum Hered*. 1981;31: 232-237.
36. Boomsma DI, Kempen HJM, Gevers Leuven JA, Havekes L, de Knijff P, Frants RR. Genetic analysis of sex and generation differences in plasma lipid, lipoprotein and apolipoprotein levels in adolescent twins and their parents. *Genet Epidemiol*. 1996; 13:49-60.
37. Lamon-Fava S, Jimenez D, Christian JC, Fabsitz RR, Reed T, Carmelli D, Castelli WP, Ordovas JM, Wilson PWF, Schaefer EJ. The NHLBI twin study: heritability of apolipoprotein A-I, B, and low density lipoprotein subclasses and concordance for lipoprotein(a). *Atherosclerosis*. 1991;91:97-106.
38. Selby JV, Newman B, Quiroga J, Christian JC, Austin MA, Fabsitz RR. Concordance for dyslipidemic hypertension in male twins. *JAMA*. 1991;265:2079-2084.
39. Haddad L, O×Dell SD, Day I.B, Whittall RA, Humphries SE. Identification of a common low density lipoprotein receptor mutation (R329X) in the south of England: complete linkage disequilibrium with an allele of microsatellite D19S394. *J Med Genet*. 1997;34:111-116.
40. Berg K, Pedersen JC. Normal DNA polymorphism at the low density lipoprotein receptor (LDLR) locus associated with serum cholesterol level. *Clin Genet*. 1988;34:306-312.
41. Schuster H, Humphries S, Rauh G, Held C, Keller CH, Wolfram G, Zöllner N. Association of DNA-haplotypes in the human LDLR gene with normal serum cholesterol levels. *Clin Genet*. 1990;38: 401-409.
42. Humphries S, Coviello DA, Masturzo P, Balcstreri R, Orecchini G, Bertolini S. Variation in the low density lipoprotein receptor gene is associated with differences in plasma low density lipoprotein cholesterol levels in young and old normal individuals from Italy. *Arterioscler Thromb*. 1991;11:509-526.
43. Gudnason V, Zhou T, Thormar K, Baehring S, Cooper J, Miller G, Humphries SE, Schuster H. Detection of the low density lipoprotein receptor gene PvuII intron 15 polymorphism using the polymerase chain reaction: association with plasma lipid traits in healthy men and women. *Clin Genet*. In press.
44. Ahn YI, Kamboh I, Aston CE, Ferrell RE, Hamman RF. Role of common genetic polymorphisms in the LDL receptor gene in affecting plasma cholesterol levels in the general population. *Arterioscler Thromb*. 1994;14:663-670.
45. Greenberg DA. Linkage analysis of "necessary" disease loci versus "susceptibility" loci. *Am J Hum Genet*. 1993;52:135-143.
46. Hulley SB, Rosenman RH, Bawol RD, Brand RJ. Epidemiology as a guide to clinical diagnosis: the association between triglyceride and coronary heart disease. *N Engl J Med*. 1980;302: 1383-1389.
47. Austin MA. Plasma triglyceride as a risk factor for coronary heart disease. *Am J Epidemiol*. 1989;129:249-259.

48. Pykalisto OJ, Smith PH, Brunzell JD. Determinants of adipose tissue lipoprotein lipase. *J Clin Invest*. 1987;56:1108-1117.
49. Garfinkel AS, Schotz MC. Lipoprotein lipase. In: Gotto AM Jr, ed. *Plasma Lipoproteins*. New York, NY: Elsevier Science Publishers; 1987:335-357.
50. Bijvoet S, Gagne SE, Moorjani S, Gagne C, Henderson HE, Fruchart J-C, Dallongeville J, Alaupovic P, Prins M, Kastelein JJP, Hayden MR. Alterations in plasma lipoproteins and apolipoproteins before the age of 40 in heterozygotes for lipoprotein lipase deficiency. *J Lipid Res*. 1996;37:640-650.
51. Sprecher DL, Harris BV, Stein EA, Bellet PS, Keilson LM, Simbartl LA. Higher triglycerides, lower high-density lipoprotein cholesterol, and higher systolic blood pressure in lipoprotein lipase-deficient heterozygotes: a preliminary report. *Circulation*. 1996;94: 3239-3245.
52. Pimstone SN, Gagne E, Gagne C, Lupien PJ, Gaudet D, Williams RR, Kotze M, Reymer PWA, Defesche JC, Kastelein JJP, Moorjani S, Hayden MR. Mutations in the gene for lipoprotein lipase: a cause for low HDL cholesterol levels in individuals heterozygous for familial hypercholesterolemia. *Arterioscler Thromb Vasc Biol*. 1995;15:1704-1712.
53. Mazzone T, Lopez C, Bergstraesser L. Modification of very low density lipoproteins leads to macrophage scavenger receptor uptake and cholesteryl ester deposition. *Arteriosclerosis*. 1987;7: 191-196.
54. Ramprasad MP, Li R, Bradley WA, Gianturco SH. Human THP-1 monocyte-macrophage membrane binding proteins: distinct receptor(s) for triglyceride-rich lipoproteins. *Biochemistry*. 1995;34: 9126-9135.
55. Suzuki H, Kurihara Y, Takeya M, Kamada N, Kataoka M, Jishage K, Ueda O, Sakaguchi H, Higashi T, Suzuki T, Takashima Z, Kawabe Z, Cynshi O, Wada Z, Honda M, Kurihara H, Aburatani H, Doi T, Matsumoto A, Akzuma S, Noda T, Toyoda Y, Itakura H, Yazaki Y, Horiuchi S, Takahashi K, Kruijt JK, van Berkel TJC, Steinbrecher UP, Ishibashi S, Maeda N, Gordon S, Kodama T. A role for macrophage scavenger receptors in atherosclerosis and susceptibility to infection. *Nature*. 1997;386:292-296.

Angiotensin-Converting Enzyme and Angiotensinogen Gene Polymorphisms and Heart Rate Variability in Twins

Andreas Busjahn, PhD, Andreas Voss, MD, Hans Knoblauch, MD, Margit Knoblauch, PhD, Eva Jeschke, Niels Wessel, Jürgen Bohlender, MD, Jesse McCarron, BS, Hans-Dieter Faulhaber, MD, Herbert Schuster, MD, Rainer Dietz, MD, and Friedrich C. Luft, MD

Decreased heart rate variability (HRV) is associated with congestive heart failure, post-myocardial infarction, ventricular arrhythmias, sudden cardiac death, and advancing age. A deletion/insertion polymorphism in the angiotensin-converting enzyme (ACE) gene and a substitution (M235T) in the angiotensinogen gene have been associated with risk for heart disease. The aim of this study was to determine the heritability of HRV and related parameters in monozygotic and dizygotic twins and to assess the influence of ACE and angiotensinogen polymorphisms. We studied 95 MZ pairs and 46 DZ pairs. We measured HRV and related parameters, ACE and angiotensinogen levels, plasma norepinephrine, ACE, and angiotensinogen genotypes. We found that HRV and related parameters were significantly influenced by genetic variability, although nonshared genetic effects were also important. Angiotensinogen and plasma norepinephrine were generally correlated with decreased HRV, whereas ACE was correlated with perturbances of normal rhythmic HRV. Nevertheless, the DD ACE genotype was associated with increased HRV ($p < 0.05$), whereas angiotensinogen polymorphisms had no effect. We conclude that HRV and related parameters are in part heritable. Interestingly, the DD ACE genotype is associated with increased HRV. ©1998 by Excerpta Medica, Inc.

(Am J Cardiol 1998;81:755–760)

Heart rate variability (HRV), defined as spontaneous fluctuations in sinus rate due to internal and external body processes, is an indicator of risk for death after a cardiac event.[1] HRV decreases with age and with certain diseases such as congestive heart failure, diabetic neuropathy, post-myocardial infarction, and in some forms of inducible ventricular tachycardia or ventricular fibrillation.[1,2] HRV parameters are determined to a great extent by baroreceptor reflex-related mechanisms, and sympathetic and parasympathetic tone, all of which deteriorate with age.[1–3] Furthermore, HRV parameters show gender-related, as well as age-related differences, suggesting additional endocrine-mediated effects.[4] Genetic variability exerts considerable influence on resting and stress-induced autonomic tone.[5] The effect of genetic influences on HRV-related parameters has not been examined. We recruited monozygotic and dizygotic normal twin subjects to study genetic effects on HRV. Because an insertion/deletion (I/D) polymorphism in intron 16 of the angiotensin-converting enzyme (ACE) gene has been associated with myocardial infarction and left ventricular hypertrophy,[6,7] and because a polymorphism in the angiotensinogen gene (M235T substitution) is associated with increased blood pressure,[8] we also determined if the ACE gene I/D alleles and the angiotensinogen M235T substitution were associated with HRV.

METHODS

We recruited 141 pairs of MZ (95 pairs) and DZ (46 pairs) twins by advertisement (print media) to participate in studies involving blood pressure and blood pressure reactivity to physical and mental stress.[9] The subjects were all German Caucasians. They were recruited from various parts of Germany, which minimizes any regional effects. The protocol was approved by the University's committee on the protection of human subjects and written informed consent was obtained from all participants. Blood was obtained for the determination of zygosity. The zygosity was verified with the use of 5 highly polymorphic short tandem repeat polymerase chain reaction-amplified microsatellite markers, namely THO1, TPOX, FES/FPS, F13A1, and FGA.[10] We used fluorescent-labeled primers in a multiplex automated genotyping system relying on a 373 DNA-sequencer and 672 GENESCAN and GENOTYPER software (all Applied Biosystems, Foster City, California). Plasma norepinephrine was measured with high-performance liquid chromatography with electrochemical detection,[11] ACE activity in plasma was determined by means of a synthetic substrate (FAPGG) as outlined elsewhere,[12] and plasma angiotensinogen was determined by radioimmunoassay.[13]

Blood pressure and heart rate were measured in the nondominant arm by an automated oscillometric

From Franz Volhard Clinic at the Max Delbück Center for Molecular Medicine, Virchow Klinikum, Humboldt University of Berlin, Berlin, Germany. This study was supported in part by a grant-in-aid from the BMBF LPD 1995; by a grant-in-aid from the Bundesministerium ü Bildung und Forschung; and by Vo 505/2-1 from the Deutsche Forschungsgemeinschaft, Bonn, Germany. Manuscript received September 5, 1997; revised manuscript received and accepted December 15, 1997.

Address for reprints: Friedrich C. Luft, MD, Franz Volhard Clinic, Wiltberg Strasse 50, 13122 Berlin, Germany.

0002-9149/98/$19.00
PII S0002-9149(97)01019-9

TABLE I Demographic, Clinical, Echocardiographic, and Angiotensin-Converting Enzyme (ACE) and Angiotensinogen Plasma Level Data in Groups With Different ACE Genotypes

Variables	II (n = 31)	ID (n = 77)	DD (n = 33)
Age (yrs)	34 ± 14	33 ± 14	34 ± 14
Gender (M/F)	6/21	18/61	10/23
Height (cm)	169 ± 8	168 ± 8	170 ± 9
Weight (kg)	66 ± 9	66 ± 13	69 ± 14
BMI (kg/m^2)	23 ± 4	23 ± 4	23 ± 5
Systolic BP (mm Hg)	123 ± 17	123 ± 15	123 ± 14
Diastolic BP (mm Hg)	75 ± 10	73 ± 11	70 ± 10
HR (beat/min)	70 ± 9	73 ± 13	73 ± 9
ACE level (U/L)‡	25 ± 11*	44 ± 18*	57 ± 25*
Angiotensinogen (μg Ang I/ml)§	1.33 ± 0.35†	1.31 ± 0.33†	1.18 ± 28†

*p <0.01; †p <0.05.
‡Group differences: II-ID, II-DD, ID-DD.
§Group differences: II-DD, ID-DD.
Ang I = angiotensin I; BP = blood pressure; BMI = body mass index; HR = heart rate.

method (Dinamap, Tampa, Florida). A detailed description of our HRV methodology and the mathematics involved in its calculation has been published.[14] Analyses follow the guidelines published recently.[15] For analysis of HRV, each subject was monitored for 4 hours with an ambulatory electrocardiographic Holter recorder (Avionics Stratascan, Del Mar Avionics, Irvine, California). All electrocardiograms were than checked and edited by a technician to remove all artifacts. The 30-minute period with the least number of artifacts was analyzed. Mean heart period refers to the average NN interval over 30 minutes. NN (successive normal beat-to-beat) intervals are the normal RR intervals in the tachogram after filtering the RR time series. We determined indexes of the time and frequency domain, where SDNN is the standard deviation of successive NN intervals, and RMSSD is the square root of the mean squared differences of successive NN intervals. Power spectral density estimates describe variance as a function of frequency. P is the total power of the frequency band 0.0 to 0.4 Hz. HF is the high-frequency power in the frequency band from 0.15 Hz up to 0.4 Hz and (ULF+VLF+LF)/P the normalized lower frequency power in the frequency band from 0.0 Hz up to 0.15 Hz. The spectra were estimated by use of the fast-Fourier transformation. These measures characterize the rhythmic variability of the heart rate. We also included measures of perturbed rhythmic HRV. PNNL10 and PNNL20 are the percentages of NN interval differences <10 and 20 ms, respectively, whereas PNN100 and PNN200 are the percentages of NN intervals >100 and 200 ms, respectively. High percentages indicate a perturbance of rhythmic HRV in either direction, i.e., fixed heart rate or large beat-to-beat (erratic) changes in heart rate. All data are normalized.

The I/D polymorphism of the ACE gene was identified with the polymerase chain reaction using a set of oligonucleotide primers flanking the polymorphic site according to the method described by Rigat et al.[16] Allele frequencies and standard binomial errors were determined by the gene counting method. Allele-specific oligonucleotide hybridization was used for the genotyping of angiotensinogen codons 174 and 235. Genomic DNA was subjected to 30 rounds of amplification using primers described elsewhere.[17] The resulting 354-base pair fragment was denatured, dot-blotted in duplicate onto nitrocellulose, and then neutralized. The filters were subsequently hybridized to the appropriate ^{32}P-labeled oligonucleotides. Only 1 member of each twin pair was included for the association testing. Selecting either twin 1 or twin 2 did not influence the results. Genotype distributions of all groups were checked for Hardy-Weinberg equilibrium and were compared with each other by chi-square and likelihood ratio methods. Relative risk figures (odds ratio statistics) and their 95% confidence intervals (CI) were calculated by using the linkage package 5.1 as outlined by Terwilliger and Ott.[18]

Statistical analysis was conducted using the SPSS program. To test for differences in the mean level of the cardiovascular measures, *t* tests for independent groups were performed. In addition to univariate methods, analysis of variance was employed as a true multivariate method for the simultaneous comparison of the 3 groups of genotypes. Post hoc comparisons between groups were done with the Scheffé test. Parameters of the quantitative genetic models were estimated by path analysis techniques using the LISREL 8 program developed by Jöreskog and Sörbom.[19] Analogous to a regression analysis, the variability of any given phenotype (P) within a population can be decomposed in additive genetic influences (A), nonadditive genetic influences (D), environmental influences shared (common) by the twins within a family (C) and random environment (E): P = aA+dD+cC+eE, with a, c, e as the estimated relative influence. For MZ and DZ the covariance of their phenotype is given by: $r_{MZ} = a^2+d^2+c^2+e^2$ and $r_{DZ} = 0.5a^2+0.25d^2+c^2+e^2$. Path analysis in twin studies can estimate additive and nonadditive (dominance) components of genetic variability (estimated as a^2 and d^2) as well as 2 environmental influences, shared (c^2) and nonshared environmental influences (e^2).[20] These values estimate the relative amount of the variable's influence on interindividual differences up to a sum of 1. Genetic as well as environmental effects were estimated by the best fitting model as selected by the chi-square value. The LISREL 8 output also gives estimates of the goodness-of-fit index, the adjusted goodness-of-fit index, and the Akaike information criterion. These estimates concurred with the results of the chi-square analysis, so we elected not to present these estimates.

RESULTS

Table I lists demographic data on all subjects divided into ACE genotypes. The ACE genotype

TABLE II Correlation Coefficients Between HRV Parameters and Blood Pressure, Heart Rate, Plasma Norepinephrine, Plasma Angiotensin-converting Enzyme Activity (ACE), and Plasma Angiotensinogen*

	Systolic Blood Pressure	Diastolic Blood Pressure	Heart Rate	Plasma Norepinephrine	ACE	Angiotensinogen
Mean heart period	−0.16	−0.39	–	−0.32	–	−0.35
SDNN	−0.25	−0.30	−0.48	−0.22	–	−0.33
RMSSD	−0.24	−0.28	−0.49	−0.27	–	−0.32
HF	−0.14	−0.14	−0.28	−0.19	–	–
Total power	−0.19	−0.24	−0.39	−0.20	–	−0.30
ULF + VLF + LF	0.20		0.15	0.14	–	–
PNNL10	0.44	0.37	0.55	0.32	0.18	0.23
PNNL20	0.43	0.39	0.56	0.34	0.15	0.28
PNN100	−0.15	−0.24	−0.45	−0.22	0.14	−0.31
PNN200	–	–	−0.24	−0.14	0.11	−0.23

*Only significant ($p < 0.05$) correlations are given.

HF = normalized power in the frequency band from 0.15 to 0.4 Hz; Mean heart period = average NN interval/30 minutes; PNNL10 and 20 = percentages of NN interval differences <10 and 20 ms, respectively; PNN100 and 200 = percentages of NN interval differences >100 and 200 ms, respectively; RMSSD = root-mean-square of NN interval successive differences; SDNN = standard deviation of filtered NN intervals; Total power = total power in the spectrum; ULF, VLF, and LF = power in the frequency band from 0 to 0.0033, 0.0044 to 0.04, and 0.04 to 0.15 Hz, respectively.

TABLE III Results for Twin Analysis (MZ = 95 pairs, DZ = 46 pairs)*

	a^2	d^2	c^2	e^2	r_{MZ}/r_{DZ}	c^2/df
Mean heart period	0.17	–	0.37	0.46	0.58/0.38	1.77/3
SDNN	–	0.60	–	0.40	0.64/0.22	4.20/4
RMSSD	–	0.65	–	0.35	0.68/0.13	3.17/4
Total power	0.56		–	0.44	0.60/0.24	0.74/4
ULF + VLF + LF	–	0.47	–	0.53	0.50/0.05	0.33/4
HF	0.39		–	0.61	0.40/0.21	1.69/4
PNNL10	–	0.56	–	0.44	0.63/0.06	10.75/4
PNNL20	–	0.59	–	0.41	0.63/0.06	10.75/4
PNN100	–	0.58	–	0.42	0.58/0.16	2.4/4

*Additive genetic factors (a^2), nonadditive genetic factors (d^2), shared environmental (c^2), and nonshared environmental (e^2) factors are given.

Only significant ($p < 0.05$) data are presented.

Abbreviations as in Table II.

had a significant effect on plasma ACE levels; the D allele conferred a higher ACE level. The ACE genotype also had an effect on angiotensinogen levels. Persons with the DD ACE genotype had lower angiotensinogen levels. Table II lists the correlation coefficients between HRV parameters and blood pressure, heart rate, plasma norepinephrine, ACE activity, and angiotensinogen in the entire cohort. Only significant ($p < 0.05$) correlations are given. Numerous correlations were observed between HRV parameters and systolic blood pressure, diastolic blood pressure, and heart rate. Plasma norepinephrine was generally inversely correlated with rhythmic HRV, PNN100, and PNN200, and directly correlated with PNNL10, PNNL20, and ULF + VLF + LF. ACE activity and angiotensinogen concentrations were directly correlated with PNNL10 and PNNL20, as well as PNN100 and PNN200. Angiotensinogen was inversely correlated with SDNN, RMSSD, and total power. The correlations are consistent with the interpretation that plasma norepinephrine, and angiotensinogen concentrations are inversely related to HRV. The ACE inhibitor, however, is directly correlated with measures of arrhythmic HRV.

Table III lists the significant heritability estimates. Additive genetic factors (a^2), nonadditive genetic factors (d^2), shared environmental (c^2), and nonshared environmental (e^2) factors are given. Only significant ($p < 0.05$) data are presented in the Table. Significant additive and nonadditive genetic factors were commonly identified. Shared environmental factors were unusual. A significant effect was found only for the mean heart period. Nonshared environmental factors were significant in every instance.

The 282 twins were genotyped according to the ACE intron deletion polymorphism. The twins met the Hardy Weinberg equilibrium criteria. When we used twin 1 of any given twin pair, 33 had the DD, 77 had ID, and 31 had II genotype. Thus, 33 had DD, whereas 108 had ID or II genotypes. Table IV lists the differences between the DD and II/ID genotypes. The standard deviation of NN intervals, the root-mean-square of NN intervals, the power in the high-frequency band, and probability of unpredictable changes in heart rate (PNN100 and PNN200) were significantly different comparing subjects with DD genotype with the II or ID genotypes. Figure 1 shows the root-mean-square of successive differences of all normal-to-normal NN intervals as a function of ACE genotypes. The value was significantly greater for the DD genotype than for II or ID genotypes. In general, these data indicate that persons with the DD genotype had greater HRV than persons with the I allele. The angiotensinogen M235T substitution was not associated with any of the HRV-related parameters.

TABLE IV Angiotensin-Converting Enzyme Genotypes and Heart Rate Variability Parameters

	II + ID (n = 108)	DD (n = 33)
Age	31.5 ± 11.4	30.1 ± 10.2
SDNN	2.1 ± 0.8	2.8 ± 1.4*
RMSSD	1.3 ± 0.7	2.2 ± 1.6*
HF	0.5 ± 0.5	1.4 ± 2.0*
Power	1.0 ± 0.8	1.6 ± 1.5
PNN100	0.47 ± 0.8	1.34 ± 1.5*
PNN200	0.13 ± 0.3	0.94 ± 2.2*

*$p < 0.05$, comparing II + ID with DD.
Values expressed as mean ± SD.
Abbreviations as in Tables II and III.

DISCUSSION

The important findings in this study are that HRV and related parameters show correlations with blood pressure, heart rate, plasma norepinephrine, angiotensinogen, and ACE levels. HRV and related parameters are heritable, and are more pronounced in persons carrying the ACE intron 16 DD genotype than in those possessing the I allele. We found no genetic association between HRV and the angiotensinogen locus. From the heritability estimates, we concluded that genetic influences on HRV were mostly dominant. Codominant effects were assessed by analysis of variance, which showed no difference between II and ID genotypes, whereas both were significantly different from the DD genotype. For this reason, we compared the DD genotype with the II and ID genotypes. To our knowledge, no earlier studies have examined genetic influences on HRV. The HRV parameters all reflect the influence of different physiologic systems (sympathetic tone, parasympathetic tone, and so forth) that are heritable to varying degrees. The range of genetic variability we observed for HRV lay between 39% to 65%; the rest could be attributed to environmental influences. Genetic influence on HRV was significantly greater than that on heart rate, which was only 17%. Common environmental effects were unusual except for heart rate. Thus, the family-related environment such as lifestyle, social economic status, etc., seemed not to have a major influence on HRV. On the other hand, the nonshared environmental effects, e.g., differences in how 1 twin slept the night before compared with the other twin, differences in illness spectrum, differences in alcohol intake, etc., had a major influence on HRV and related parameters. We did not measure these environmental effects precisely and are not able to speculate on which were important.

Several studies in humans and experimental animals demonstrate convincingly that impaired baroreceptor reflex sensitivity and reduced HRV increases the risk of cardiac mortality.[21] Particularly, the low-frequency spectral component of HRV, rather than a reduced high-frequency component, are associated with an increased risk of life-threatening arrhythmia after myocardial infarction.[22,23] Although HRV, assessed by both time and frequency domain parameters, is very stable and reproducible over shorter periods of time, HRV decreases with advancing age.[2–4] Impaired cardiovascular autonomic regulation, as reflected by HRV, may be partly responsible for the greater mortality after myocardial infarction observed in the elderly. Plasma norepinephrine concentrations increase with advancing age.[24] Thus, we were interested in the fact that plasma norepinephrine was inversely correlated with HRV. The same was true for angiotensinogen. A long-term association between angiotensin II and sympathetic activation has been suggested.[25] These findings may be relevant to the previously described decrease in HRV with increasing age and the greater degree of mortality after myocardial infarction in the elderly.

We are not able to explain why the ACE DD genotype would be associated with higher HRV. Persons with the D allele had higher ACE levels. We were recently able to show that the ACE gene locus is responsible for almost all of the genetic variance in ACE levels.[26] We found that ACE levels were correlated with perturbed rhythmic HRV, namely PNNL10, PNNL20, PNN100, and PNN200. However, we found no correlation between all measures of rhythmic HRV and ACE levels. We did observe an inverse correlation between ACE levels and angiotensinogen concentrations. This finding suggests that higher ACE activity may result in a greater degree in angiotensinogen consumption, consistent with the notion that ACE is a rate-limiting step in angiotensin II generation.[27] Angiotensinogen itself was inversely correlated with rhythmic HRV. Taken together, these observations are consistent with a functional effect of the DD genotype on rhythmic HRV via renin-angiotensin–mediated influences.

Conceivably, 2 pathways may be operative: 1 may involve the renin-angiotensin system, while another could involve the kinin system. Bradykinin, which is degraded by ACE, reduces blood pressure and increases heart rate.[28] Finally, there is a substantial interaction between the renin-angiotensin system and the baroreceptor reflex, which is under sympathetic and parasympathetic nervous system control. We found a significant interaction between ACE activity and plasma norepinephrine. ACE levels were correlated not only with PNNL10 and PNNL20, which indicate periods of inflexible heart rate, but also with PNN100 and PNN200, which indicate larger unpredictable beat-to-beat variations in heart rate. The direction of these changes are dependent on plasma norepinephrine values, because plasma norepinephrine is directly correlated with PNNL10 and PNNL20, and inversely correlated with PNN100 and PNN200. These results are consistent with earlier observations in humans.[29]

The ACE DD genotype, which has been associated with risk for myocardial infarction[6] and left ventricular hypertrophy,[7] is also associated with longevity. Schächter et al[30] studied 338 adults aged ≥100 years and compared these subjects with

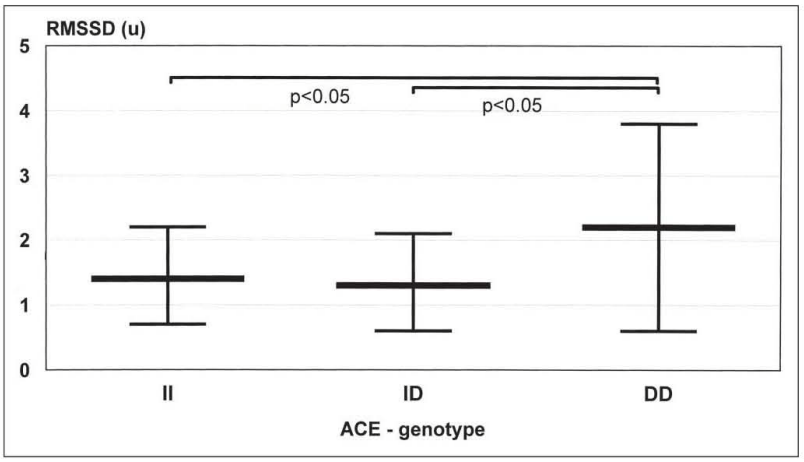


FIGURE 1. Relation between root-mean-square of the NN interval successive differences (RMSSD) (ms) and angiotensin-converting enzyme (ACE) I/D genotypes. The ACE I allele was associated with lower values, indicating a lower heart rate variability. The ACE DD genotype had significantly higher RMSSD values than the genotypes containing the I allele.

adults aged 20 to 70 years. They found that the ACE DD genotype was significantly increased among centenarians compared with the younger control cohort and with the expected genotype distribution. We were able to confirm these findings in a sample of 349 Berliners aged >80 years (unpublished observations). These results imply that persons with the ACE DD genotype, providing they avoid the pitfalls of possible early increased cardiovascular risk, may be more likely to be among the "old" elderly than those with the I allele. Our observation that persons with the DD genotype have greater HRV may explain the unexpected finding that an ACE variant, which may predispose to coronary heart disease, is unexpectedly frequent in French centenarians and German octogenarians. We suggest that the ACE gene may exert pleiotropic, age-dependent effects on longevity.

1. Kleiger RE, Miller JP, Bigger JT, Moss AJ. Decreased heart rate variability and its association with increased mortality after acute myocardial infarction. *Am J Cardiol* 1987;59:256–62.

2. Schwartz PJ, Stone HL. The role of the autonomic nervous system in sudden coronary death. *Ann NY Acad Sci* 1982;382:162–180.

3. Copie X, Hnatkova K, Staunton A, Fei L, Camm AJ, Malik M. Predictive power of increased heart rate versus depressed left ventricular ejection fraction and heart rate variability for risk stratification after myocardial infarction. *J Am Coll Cardiol* 1996;27:270–276.

4. Huikuri HV, Pikkujämsä SM, Airaksinen J, Ikäheimo MJ, Rantala AO, Kauma H, Lilja M, Kesäniemie A. Sex-related differences in autonomic modulation of heart rate in middle-aged subjects. *Circulation* 1996;94:122–125.

5. Rose RJ. Genes, stress, and cardiovascular reactivity. In: Turner JR, Sherwood A, Light KC, eds. Individual Differences in Cardiovascular Response to Stress. New York, London: Plenum Press, 1992:87–102.

6. Cambien F, Costerousse O, Tiret L, Poirier O, Lecerf L, Gonzales MF, Evans A, Arveiler D, Cambou JP, Luc G, Rakotovao R, Ducimetiere P, Soubrier F, Alhenc-Gelas F. Plasma level and gene polymorphism of angiotensin-converting enzyme in relation to myocardial infarction. *Circulation* 1994;90:669–676.

7. Schunkert H, Hense H-W, Holmer SR, Stender M, Perz S, Keil U, Lorell BH, Riegger GAJ. Association between an deletion polymorphism of the angiotensin-converting-enzyme gene and left ventricular hypertrophy. *N Engl J Med* 1994; 330:1634–1638.

8. Jeunemaitre X, Soubrier F, Kotelevtsev YV, Lifton RP, Williams CS, Charru A, Hunt SC, Hopkins PN, Williams RR, Lalouel J-M, Corvol P. Molecular basis of human hypertension: role of angiotensinogen. *Cell* 1992;71:169–180.

9. Busjahn A, Faulhaber H-D, Viken RJ, Rose RJ, Luft FC. Genetic influences on blood pressure with the cold pressor test: a twin study. *J Hypertens* 1996;14: 1195–1199.

10. Hammond HA, Jin L, Zhong Y, Caskey CT, Chakraborty R. Evaluation of 13 short tandem repeat loci for use in personal identification applications. *Am J Hum Genet* 1994;55:175–189.

11. Eisenhofer G, Goldstein DS, Stull R, Keiser HR, Sunderland T, Murphy DL. Simultaneous liquid chromatographic determination of 3,4-dihydroxyphenylglycol, catecholamines, and 3,4-dihydroxyphenylalanine in plasma and their responses to inhibition of monoamine oxidase. *Clin Chem* 1986;32: 2030–2033.

12. Beneteau B, Baudin B, Morgant C, Giboudeau J, Baumann, FC. Automated kinetic assay of angiotensin-converting enzyme in serum. *Clin Chem* 1986;32: 884–886.

13. Bohlender J, Ménard J, Wagner J, Luft FC, Ganten D. Human renin-dependent hypertension in transgenic hAOGEN rats. *Hypertension* 1996;27:535–540.

14. Voss A, Kurths J, Kleiner HJ, Witt A, Wessel N, Saparin P, Osterziel KJ, Schurath R, Dietz R. The application of methods of non-linear dynamics for the improved and predictive recognition of patients threatened by sudden cardiac death. *Cardiovasc Res* 1996;31:419–433.

15. Task Force of the European Society of Cardiology and the North American Society of Pacing and Electrophysiology. Heart rate variability: standards of measurement, physiological interpretation, and clinical use. *Circulation.* 1996; 93:1043–1065.

16. Rigat B, Hubert C, Alhenc Gelas F, Cambien F, Corvol P, Soubrier F. An insertion/deletion polymorphism in the angiotensin 1-converting enzyme gene accounting for half the variance of serum enzyme levels. *J Clin Invest* 1990;86: 1343–1346.

17. Ward K, Hata A, Jeunemaitre X, Helin C, Nelson L, Namikawa C, Farrington PF, Ogasawara M, Suzumori K, Tomoda S, Berrebi S, Sasaki M, Corvol P, Lifton RP, Lalouel J-M. A molecular variant of angiotensinogen associated with pre-eclampsia. *Nature Genet* 1994;4:59–61.

18. Terwilliger JD, Ott J. Handbook of Human Genetic Linkage. Baltimore, London: Johns Hopkins University Press, 1994.

19. Jöreskog KG, Sörbom D. LISREL 8.-Scientific Software International Inc. Chicago IL: 1993.

20. Neale MC, Cardon LR. Methodology for Genetic Studies of Twins and Families. Netherlands: Kluwer Academic Publishers, Dordrecht, 1992:496–522.

21. Tsuji H, Venditti F Jr, Manders ES, Evans JC, Larson MG, Feldman CL, Levy D. Reduced heart rate variability and mortality risk in an elderly cohort. *Circulation* 1994;90:878–883.

22. Hohnloser SH, Klingenheben T, van de Loo A, Gablawetz E, Just H,

Schwartz PJ. Reflex versus tonic vagal activity as a prognostic parameter in patients with sustained ventricular tachycardia or ventricular fibrillation. *Circulation* 1994;90:1068–1073.
23. Huikuri HV, Koistinen MJ, Yli-Mäyry S, Airaksinen KEJ, Seppänen T, Ikäheimo MJ, Myerburg RJ. Impaired low-frequency oscillations of heart rate in patients with prior acute myocardial infarction and life-threatening arrythmias. *Am J Cardiol* 1995;76:56–60.
24. Ziegler MG, Lake CR, Kopin IJ. Plasma noradrenaline increases with age. *Nature* 1976;261:333–335.
25. Brooks VL, Osborn JW. Hormonal-sympathetic interactions in long-term regulation of arterial blood pressure: an hypothesis. *Am J Physiol* 1995;268: R1343–R1358.
26. Busjahn A, Knoblauch J, Knoblauch M, Schuster H, Bohlender J, Faulhaber H-D, Luft FC. Angiotensin converting enzyme and angiotensinogen gene polymorphisms, plasma levels, and left ventricular size: a twin study. *Hypertension* 1997;29:165–170.
27. Müller DN, Bohlender J, Hilgers KF, Dragun D, Costerousse O, Ménard J, Luft FC. Vascular expression of the angiotensin converting enzyme gene regulates the rate of local angiotensin II formation. *Hypertension* 1997;29:98–104.
28. Fargeas J, Fargeas MJ, Ruckebusch Y. Abatement and arrest of the heart following intracarotid injection of bradykinin in dogs. *J Physiol Paris* 1969; 61(suppl 1):128–129.
29. Tulen JH, Man in't Veld AJ, Van Roon AM, Moleman P, Van Steenis HG, Blankestijn PJ, Boomsma F. Spectral analysis of hemodynamics during infusions of epinephrine and norepinephrine in men. *J Appl Physiol* 1994;76:1914–1921.
30. Schächter F, Faure-Delanef L, Guenot F, Rouger H, Froguel P, Lesueur-Ginot L, Cohen D. Genetic associations with human longevity at the APOE and ACE loci. *Nature Genet* 1994;6:29–32.

Chymase Gene Locus Is Not Associated With Myocardial Infarction and Is Not Linked to Heart Size or Blood Pressure

Arne Pfeufer, MS, Andreas Busjahn, PhD, Athanasios Vergopoulos, MD, Hans Knoblauch, MD, Hidenori Urata, MD, Karl Josef Osterziel, MD, Marianne Menz, MD, Thomas F. Wienker, MD, Hans Dieter Faulhaber, MD, Armin Steinmetz, MD, Herbert Schuster, MD, Rainer Dietz, MD, and Friedrich C. Luft, MD

The insertion/deletion polymorphism in the angiotensin-converting enzyme (ACE) gene influences plasma ACE levels and is associated with a risk for myocardial infarction, left ventricular hypertrophy, and severity of disease in hypertrophic cardiomyopathy.[1] ACE is not the only enzyme responsible for the conversion of angiotensin (Ang) I to Ang II. In the human heart, another enzyme heart chymase (CMA) is capable of converting Ang I to Ang II.[2] CMA is the most efficient and specific Ang II forming enzyme described thus far.[3] CMA-related Ang II formation could be responsible for failure of left ventricular hypertrophy to regress with ACE inhibitor treatment, as well as for the disappointing results of ACE inhibitor treatment to avoid recurrence after percutaneous transluminal angioplasty.[4] We reported earlier that in patients with hypertrophic cardiomyopathy, the DD allele of ACE was associated with increased expression of the disease, but were unable to establish an association for a polymorphism in the CMA gene.[5] We now looked for an effect of CMA polymorphisms on the risk for myocardial infarction, on heart size, and blood pressure in different populations. We purposely studied a population in which we had already found an association between the DD genotype of ACE and risk for myocardial infarction as a positive control.[6] Similarly, we examined monozygotic and dizygotic twins in whom we had already found an influence of the I/D alleles on ACE plasma levels and on heart size.[7]

• • •

We studied the bi-allelic CMA/B polymorphism in 394 consecutive patients who had undergone cardiac catheterization because of known or suspected coronary artery disease in whom we reported ACE I/D genotypes earlier.[6] Of these patients, 165 had had acute myocardial infarction (AMI) as documented by clinical, electrocardiographic, and enzyme criteria. In these patients with AMI, angiographic evidence of coronary artery disease was present in every instance. The 229 AMI-negative patients had had no clinical, electrocardiographic, or enzymatic evidence of AMI. All underwent echocardiography as well and none had evidence for other forms of heart disease. Patients without electrocardiographic or enzyme changes suggestive of AMI who nonetheless had dyskinetic segments were not included in the study. Most patients (>90%) had evidence of coronary artery disease, whereas some did not. We also determined CMA/B genotypes in 104 normal persons to determine the allele frequencies of this polymorphism. Finally, we confirmed the Mendelian segregation of the CMA/B polymorphism in 4 pedigrees with 18 informative meioses as described earlier.[6]

Sixty-six pairs of dizygous (DZ) and 100 pairs of monozygous (MZ) twins from our clinical research unit sibling-pair investigation program were included in the study. All twins were characterized by detailed history and physical examination and underwent echocardiography by a single investigator who was unaware of the genotyping data. Blood pressure was determined oscillometrically (Dinamap, Tampa, Florida). The same subjects were included in an investigation that demonstrated an association between heart size and the ACE I/D polymorphism.[7] All protocols were approved by our committee for the protection of human subjects. After written informed consent was obtained, we obtained blood samples and prepared DNA for genetic analysis. Genotype distributions of all groups were checked for Hardy-Weinberg equilibrium and were compared to each other by chi-square and likelihood ratio methods. Mean differences were determined by analysis of variance.

The CMA gene CMA1 is located at chromosomal position 14q11.2 within a cluster of several serine protease genes, among them cathepsin G (CTSG), granzyme B (CTLA1), and granzyme H (CTSGL2).[8] The order of the genes is cen-CTLA1-CTLA2-CTSG-CMA1-tel. In the CMA gene exist 2 known biallelic markers CMA/A and CMA/B.[9] For analysis of association with CMA/B, the 394 patients were genotyped for this polymorphism. In brief, a 285 bp PCR reaction product was amplified with the primers CMA 1.3F 5′-GGAAATGTGAGCAGATAGTGCAGTC-3′ and CMA 1.3R 5′-AATCCGGAGCTGGAGAACTCTT-GTC-3′. The product was digested with BstXI. A alleles of CMA/B remained uncut, while G alleles produced 2 bands at 195 and 95 bp.

The 66 DZ twin pairs and their parents were analyzed for their identity by descent (IBD) status in the CMA1 locus. To increase the information in this analysis, we identified a composite microsatellite in the 3′UTR of CMA1, which consisted of a (TG)n repeat

From the Franz Volhard Clinic and Max Delbrück Center for Molecular Medicine, Virchow Klinikum, Humboldt University of Berlin, Berlin, Germany. This study was supported by a grant-in-aid from the Bundesministerium für Bildung und Forschung, Bonn, Germany. Dr. Luft's address is: Franz Volhard Clinic, Wiltberg Strasse 50, 13125 Berlin, Germany. Manuscript received May 11, 1998, and accepted May 17.

TABLE I Observed CMA/B Genotype Frequencies in the Case-Control Sample

	No.	CMA/B Genotype		
		AA	AG	GG
No CAD	110	36 (0.33)	50 (0.45)	24 (0.22)
CAD	119	32 (0.27)	60 (0.50)	27 (0.23)
AMI	165	59 (0.36)	66 (0.40)	40 (0.24)

TABLE II Demographic Data, Blood Pressure Values (mean ± SD), Correlations (r), and Heredity Estimates (a2)

	MZ Twins (n = 200)	DZ Twins (n = 132)	a2 (rMZ/rDZ)
Age (yr)	29 ± 12	31 ± 12	
Men/women	52/148	85/47	
Height (cm)	169 ± 8	170 ± 8	
Weight (kg)	65 ± 11	67 ± 12	
BMI (kg/m)	22.4 ± 3.5	22.8 ± 3.4	
Systolic BP sitting (mm Hg)	118 ± 11	118 ± 10	0.81 (0.81/0.31)
Diastolic BP sitting (mm Hg)	69 ± 9	71 ± 8	0.41 (0.80/0.59)
Posterior wall thickness (mm)	8.7 ± 1.6	8.6 ± 1.6	0.26 (0.48/0.26)
Septum (mm)	8.9 ± 1.7	8.8 ± 1.6	0.37 (0.64/0.37)

BMI = body mass index; BP = blood pressure.

TABLE III Linkage Analysis for Cardiac Size and Blood Pressure Corrected for Age and Gender, and the Microsatellite Markers*

Phenotype	Marker	Slope	p Values
Posterior wall thickness	D14S264	−4.4	0.20
	D14S590	−0.3	0.48
Septum	D14S264	−2.3	0.07
	D14S590	−0.6	0.36
Systolic BP	D14S264	−76	0.19
	D14S590	−43	0.30
Diastolic BP	D14S264	−8	0.45
	D14S590	−35	0.31

*No significance was found.
BP = blood pressure.

termed CMA/C followed by a G-rich stretch of 10 bases and an (AG)n repeat termed CMA/D in the orientation of the coding strand. The sequence of the compound microsatellite we discovered in the 3′UTR of the chymase gene is: 5′ AAGACACAGCTTCTA GTCGTGAGTGTGTGTCCCTCTCTGCTGCTCTCT TCTCCTGCACATGTGACCTGATTCCCAGCCCAA GCACCAAGGATTTGGAAGGGTGTGTGTGTGTG TGTGTGTGTGTGTGTGTGTGTGTGTGTATGGGG GGGGAGGGAGAGAGAGAGAGAGAGAGAAAT AGAGAGGAATTAATGAAGAAAAAAAAA-3′. The repeats CMA/C and CMA/D are underlined. The DZ twins were genotyped for the markers CMA/B, CMA/C, CMA/E, D14S264, and D14S590 according to methods described earlier.[5]

For the linkage study, the DZ pairs were selected and used as ordinary sib pairs, but with the advantage of perfect age matching and reduced environmental variation affecting the phenotype. The power of the twin model in elucidation of complex genetic disease has recently been emphasized by Martin et al.[10] The MZ twins were used to estimate allele frequencies for the markers tested. The zygosity was verified.[11] Linkage analyses were performed using the SIBPAL program of statistical analysis for genetic epidemiology (SAGE) package.* The underlying basis for the sibling-pair linkage approach is to analyze the difference in a quantitative trait between siblings as a function of the number of marker alleles they share that are identical by IBD status. For each sibling pair and each locus, the proportion of alleles IBD, based on parental genotypes, is calculated as well as the squared trait difference. To test for linkage, a linear regression analysis is performed with the squared trait difference as the dependent variable and IBD as the independent variable. Sibling-pair analysis to determine linkage does not require the specification of a genetic model. The underlying trait can follow either Mendelian or non-Mendelian modes of inheritance.

Table I shows the CMA/B genotyping results of 394 patients undergoing coronary angiography. In all, 110 patients with normal coronary angiograms and no coronary artery disease, 119 patients with coronary artery disease but no myocardial infarction, and 165 patients with coronary artery disease with documented AMI are given. Genotyping revealed Hardy Weinberg equilibrium in the entire group and in all subgroups. Genotype frequencies of CMA/B genotypes AA, AG, or GG between patients either with or without CAD (groups CAD and AMI vs no CAD) and between patients with or without AMI (group AMI vs CAD and no CAD) were compared. To analyze for a putative weak effect that might synergistically influence both development of CAD and progression to AMI, we directly compared patients with AMI to those without CAD (group AMI vs no CAD). There was no significant association of any CMA/B genotype with any of these phenotypes.

Demographic and clinical data on MZ and DZ twins is given in Table II. All subjects were in the physiologic range of cardiac size as well as blood pressure. Total genetic influence on the phenotypes (heredity) was higher for blood pressure than for cardiac size. Results of the linkage analysis is given in Table III. There was no evidence of genetic variance for cardiac size or blood pressure due to allelic variants of the CMA gene.

• • •

The important findings in this study are that CMA polymorphisms were not associated with risk for myocardial infarction in a cohort in which such a risk had

*S.A.G.E. Statistical Analysis for Genetic Epidemiology, Release 2.2. Computer program package available from the Department of Epidemiology and Biostatistics, Case Western Reserve University, Cleveland, Ohio, 1996.

been shown for the ACE gene.[6] Second, no effect of CMA polymorphisms could be shown on posterior wall thickness, even though in the same subject group such an effect had been shown for the ACE gene.[7] Finally, we saw no effect of CMA polymorphisms on blood pressure in that same group. Interestingly, in the twins, the ACE DD genotype was associated with significantly lower diastolic blood pressure than the ID or II genotypes.[7] Thus, our data do not support a major role for genetic variance on CMA in influencing phenotypes attributed to Ang II in the heart, namely risk of AMI, effect on blood pressure, or on cardiac dimensions. We had observed earlier that CMA polymorphisms had little or no effect on heart size in patients with hypertrophic cardiomyopathy.[5]

Our findings have 2 possible explanations. Either the CMA locus and its gene product CMA do not significantly contribute to the pathogenesis of the investigated phenotypes, myocardial infarction, coronary disease, blood pressure, and heart size, or there are no sufficiently variant alleles present in the CMA locus that would exercise different effects on CMA activity to permit clinical detection. Because our study included not only association but also a linkage approach, we are confident that we have not overlooked a possible effect. These results do not permit the conclusion that CMA is not relevant to the production of Ang II and its effects in the human heart or blood vessels. It is possible that we failed to examine the relevant phenotypes important for CMA. For instance, Nishimura et al[4] emphasized an important role for ACE-independent Ang II production in vascular remodeling after balloon dilatation. We did not examine such phenotypes in our studies. However, we were unable to show a significant effect of the CMA gene locus on AMI, coronary disease, blood pressure, or heart size.

In conclusion, although CMA may convert Ang I to Ang II in the human heart, we were unable to identify an association between allelic variants of CMA and AMI, nor were we able to demonstrate linkage between the CMA gene locus and heart size. These findings contrast with those of the ACE gene in the same subject groups studied.

1. Schunkert H. Polymorphism of the angiotensin-converting enzyme gene and cardiovascular disease. *J Mol Med* 1997;75:867–875.
2. Urata H, Boehm KD, Philip A, Kinoshita A, Gabrovsek J, Bumpus FM, Husain A. Cellular localization and regional distribution of an angiotensin II-forming cymase in the heart. *J Clin Invest* 1993;91:1269–1281.
3. Urata H, Kinoshita A, Misono KS, Bumpus FM, Husain A. Identification of a highly specific chymase as the major angiotensin II-forming enzyme in the human heart. *J Biol Chem* 1990;265:22348–22357.
4. Nishimura H, Hoffmann S, Balatu O, Sugimura K, Ganten D, Urata H. Angiotensin I converting enzyme and chymase in cardiovascular tissues. *Kidney Int* 1996;49(suppl 55):S18–S23.
5. Pfeufer A, Osterziel KJ, Urata H, Borck G, Schuster H, Wienker TF, Dietz R, Luft FC. Angiotensin-converting enzyme and heart chymase gene polymorphisms in hypertrophic cardiomyopathy. Am J Cardiol 1996;78:362–364.
6. Schuster H, Wienker TF, Stremmler U, Noll B, Steinmetz A, Luft FC. A variant of the angiotensin converting enzyme gene is associated with myocardial infarction, but not with risk factors or severity of coronary artery disease. Am J Cardiol 1995;76:601–603.
7. Busjahn A, Knoblauch J, Knoblauch M, Bohlender J, Menz M, Faulhaber H-D, Becker A, Schuster H, Luft FC. Angiotensin converting enzyme and angiotensinogen gene polymorphisms, plasma levels, and left ventricular size: a twin study. *Hypertension* 1997;29:165–170.
8. Caughey GH, Schaumberg TH, Zerwek EH, Butterfield JH, Hanson RD, Silverman GA, Ley TJ. The human mast cell chymase gene (CMA1): mapping to the cathepsin G/granzyme gene cluster and lineage restricted expression. *Genomics* 1993;15:614–620.
9. Heusel JW, Hanson RD, Silverman GA, Ley TJ. Structure and expression of a cluster of human hematopoietic serine protease genes found on chromosome 14q11.2. *J Biol Chem* 1991;226:6152–6158.
10. Martin N, Boomsma D, Machin G. A twin-pronged attack on complex traits. *Nat Genet* 1997;17:387–392.
11. Becker A, Busjahn A, Faulhaber H-D, Bähring S, Schuster H, Luft FC. Automated zygosity determination with microsatellites. *J Reproductive Med* 1997;42:260–266.

Usefulness of *Abciximab* for Treatment of Early Coronary Artery Stent Thrombosis

Ivan P. Casserly, MBBCh, David Hasdai, MD, Peter B. Berger, MD, David R. Holmes, Jr., MD, Robert S. Schwartz, MD, and Malcolm R. Bell, MBBS

Abciximab is a novel platelet glycoprotein IIb/IIIa receptor inhibitor with proven efficacy in the management of ischemic coronary syndromes.[1–6] Because early stent thrombosis is primarily mediated by platelet thrombosis,[7,8] administration of a platelet glycoprotein IIb/IIIa inhibitor such as abciximab could potentially be an effective method of recanalizing stents occluded with thrombus and inhibiting further thrombus formation. Our approach to treating early stent thrombosis since July 1995 has been to use adjunctive abciximab whenever possible. This study describes the angiographic and clinical outcome of patients with early stent thrombosis treated with abciximab versus patients who had not received abciximab in the treatment of early stent thrombosis.

• • •

Using the Mayo Clinic coronary intervention database, we identified all patients with stent thrombosis between July 1995 and June 1996. Stent thrombosis was defined clinically as the sudden onset of chest pain associated with ischemic electrocardiographic changes in the distribution of the stented target vessel. Early stent thrombosis was defined as thrombosis occurring between completion of the procedure and 30 days after stent insertion. Angiographically, stent thrombosis was defined as intraluminal filling defects resulting in either complete or partial vessel occlusion.

From the Division of Internal Medicine and Cardiovascular Diseases, Mayo Clinic and Foundation, Rochester, Minnesota. Dr. Bell's address is: Mayo Clinic, 200 First St. SW, Rochester, Minnesota 55905.

Manuscript received February 1, 1998; revised manuscript received and accepted May 15, 1998.

0002-9149/98/$19.00 PII S0002-9149(98)00519-0

QT Interval Is Linked to 2 Long-QT Syndrome Loci in Normal Subjects

Andreas Busjahn, PhD; Hans Knoblauch, MD; Hans-Dieter Faulhaber, MD; Thomas Boeckel, MD; Magda Rosenthal; Regina Uhlmann; Margret Hoehe, MD; Herbert Schuster, MD; Friedrich C. Luft, MD, FRCP (Edin)

Background—The rate-corrected QT interval (QTc) is heritable, and the discovery of quantitative trait loci that influence the QTc would be an important step in identifying the genes responsible for life-threatening arrhythmias in the general population. We studied 66 pairs of unselected normal dizygotic (DZ) twin subjects and their parents in a sib-pair analysis. We tested for linkage of gene loci harboring genes known to cause the long-QT syndrome (LQT) to the quantitative trait QTc.

Methods and Results—We found genetic variance on QRS duration, QRS axis, T-wave axis, and QTc. Women had a longer QTc than men. Microsatellite markers were tested in the vicinity of the gene loci for the 5 known LQT genes. We found significant linkage of QTc with the loci for LQT1 on chromosome 11 and LQT4 on chromosome 4 but not to LQT2, LQT3, or LQT5. We also found linkage of the QRS axis with LQT2 and LQT3.

Conclusions—We suggest that these quantitative trait loci may represent the presence of variations in LQT genes that could be important to the risk for rhythm disturbances in the general population. **(*Circulation*. 1999;99:3161-3164.)**

Key Words: molecular biology ■ long-QT syndrome ■ intervals ■ genetics ■ electrocardiography

Heart failure is common in the general population and is associated with sudden cardiac death from arrhythmias.[1] The chance for a normal person to develop heart failure at some time in their life is considerable. The discovery of genes responsible for the long-QT syndrome (LQT) has enabled the understanding of molecular mechanisms involved in fatal arrhythmias.[2] Thus far, 4 such genes are known, and a fifth gene locus has been identified on chromosome 4. It is conceivable but not yet shown that these genes may contain lesser functionally important variants that could contribute to rhythm disturbances in the general population. The rate-corrected QT interval (QTc) is known to be influenced by genetic variance.[3] The existence of highly polymorphic microsatellite markers enables testing of the hypothesis of whether or not LQT gene loci are linked to QTc in normal persons. We relied on normal monozygotic (MZ) and dizygotic (DZ) twin subjects to address this issue.

Methods

We recruited 166 pairs of twins (100 MZ and 66 DZ) by advertisement to participate in studies involving blood pressure regulation and cardiovascular phenotypes.[4,5] The subjects were all white Germans recruited from various parts of Germany. The protocol was approved by the University's committee on the protection of human subjects, and written informed consent was obtained from all participants. Blood was obtained for determination of zygosity and other molecular genetic studies from all the twins and the parents of the DZ twins. Each participant underwent a medical history and physical examination. None had a family history of chronic medical illness. Blood pressure was measured by a trained physician (2 measurements, 1 minute apart) with a standardized mercury sphygmomanometer, with the subject seated for 5 minutes. The mean of the 2 measurements was used. Subjects underwent echocardiography and planar ECG. A standard 12-lead ECG was performed (CARDIOVITS CS-100, Schiller AG). Duration of the QTc and RR intervals was measured in lead II. QTc was determined according to Bazett's formula.[6] ECG parameters were scored by a computer and stored for subsequent retrieval.

For this linkage study, the DZ pairs were selected and used as ordinary sib pairs but with the advantage of perfect age matching and reduced environmental variation affecting the phenotype. The power of the twin model in elucidation of complex genetic disease has recently been emphasized by Martin et al.[7] The MZ twins were used to estimate allele frequencies for the markers tested. Zygosity was verified with the use of 5 polymerase chain reaction–amplified microsatellite markers, as described in detail elsewhere.[8] We examined 2 microsatellite markers at the LQT1 locus, 3 at the LQT2 locus, 3 at the LQT3 locus, 5 at the LQT4 locus, and 3 at the LQT5 locus, as shown in Table 1.

We assessed linkage for QTc as a continuous trait.[9] Sib-pair analysis to determine linkage does not require specification of a genetic model. The underlying trait can follow either mendelian or nonmendelian modes of inheritance. Analysis was done by use of a structural equation modeling (SEM) approach,[10] as implemented in the MX package.[11] This approach is based on variance (VAR)-covariance (COV) matrices of sibs weighted by the probability of sharing 0, 1, or 2 alleles identical by descent (IBD). Phenotypic variance was decomposed into variance due to genetic background (A), variance

Received December 15, 1998; revision received March 29, 1999; accepted March 30, 1999.

From the Franz Volhard Clinic and Max Delbrück Center for Molecular Medicine, Medical Faculty of the Charité, Humboldt University of Berlin, Germany.

Correspondence to Friedrich C. Luft, Franz Volhard Clinic, Wiltberg Strasse 50, 13122 Berlin, Germany. E-mail luft@fvk-berlin.de

Circulation **is available at http://www.circulationaha.org**

TABLE 1. Microsatellite Markers Used in Linkage Analysis

Locus	Marker
LQT1	D11S1318
	D11S4146
LQT2	D7S505
	D7S636
	D7S483
LQT3	D3S1298
	D3S1260
	D3S1100
LQT4	D4S1570
	D4S1564
	D4S402
	D4S1615
	D4S429
LQT5	D21S219
	D21S65
	D21S1252

due to the quantitative trait loci (QTL) effect (Q), and environmental variance (E):

$$VAR = A^2 + Q^2 + E^2$$

For the 3 possible IBD states (sharing 0, 1, or 2 alleles), covariance of a sib pair was then defined by

$$COV_{IBD0} = 0.5A^2 \quad COV_{IBD1} = 0.5A^2 + 0.5Q^2 \quad COV_{IBD2} = 0.5A^2 + Q^2$$

To improve estimates of total variance and genetic background, MZ twins were included in the analysis, with the covariance defined as

$$COV_{MZ} = A^2 + Q^2$$

To test for a QTL effect, the difference in model fit for models with and without a QTL effect was calculated as a χ^2 statistic. For each sib pair and each locus, the proportion of alleles IBD, based on parental genotypes and independent allele-frequency estimates, was calculated with a multipoint approach as implemented in MAPMAKER/SIBS.[12] The higher power of the variance-covariance–based analysis compared with the squared trait differences–based approach by the Elston method[13] has been shown in a recent simulation study.[14] Because we used a candidate gene approach, we accepted $P<0.01$ to test for significant linkage, in accordance with the criteria defined by Lander and Kruglyak.[15]

Parameters of the quantitative genetic models were estimated by SEM with the MX program developed by Neale.[12] The variability of any given phenotype (P) within a population can be decomposed into genetic influences (A), environmental influences shared by the twins within a family (C), and effects of random environment (E):

$$P = aA + cC + eE$$

with a, c, and e as the estimated relative influence. For MZ and DZ, the covariance of their phenotype is given by

$$Cov_{MZ} = a^2 + c^2 \quad \text{and} \quad Cov_{DZ} = 0.5a^2 + c^2$$

Heritability analysis in twin studies can estimate additive components of genetic variability (estimated as a^2) as well as 2 environmental influences, shared (c^2) and nonshared (e^2) environmental influences.[16] These values estimate the relative amount of the influence on interindividual differences up to a sum of 1. Genetic as well as environmental effects were estimated by the best-fit model as selected by the χ^2 value. Statistical analysis was conducted with the SPSS program. Adjustment of phenotypic values for sex and age was done by multiple linear regression with the unstandardized residuals as the corrected phenotypes. In case of significant deviations from a normal distribution, the appropriate transformations were applied before analysis.

TABLE 2. Demographic Data and ECG Parameters in MZ and DZ Twin Subjects

Variable	MZ (mean±SD)	DZ (mean±SD)
n (pairs)	100	66
Age, y	34±15	34±12
Sex (male/female)	84/166	48/102
Height, cm	169±9	170±9
Weight, kg	67±12	70±14
Systolic blood pressure, mm Hg	125±16	123±13
Diastolic blood pressure, mm Hg	73±11	73±10
Body mass index, kg/m^2	23±4	24±4
P, ms	110±11	106±12*
PR, ms	152±20	151±22
QRS, ms	99±10	97±11
QTc, ms	414±25	416±25
P axis, °	44±20	43±21
QRS axis, °	55±25	49±26
T axis, °	37±18	33±16

*$P<0.01$.

Results

Demographic data, blood pressure values, and heredity estimates of blood pressure and ECG variables in 200 MZ and 132 DZ twins are given in Table 2. There were no significant differences between MZ and DZ twins for any of the demographic variables examined. P-wave duration was slightly longer in MZ than in DZ twins. Women had a longer QTc than men (421±25 and 405±24 ms, respectively; $P<0.001$). Table 3 gives genetic analysis showing genetic, shared environmental, and nonshared environmental effects on ECG parameters. P-wave duration, QRS duration, QTc, QRS axis, and T-wave axis showed strong genetic effects. RR interval, P-wave duration, PR interval, and P-wave axis showed shared environmental effects, and all ECG parameters showed evidence of nonshared environmental effects. Table 4 contains linkage analysis results for the tested loci in the DZ twins. Strong inference for linkage was found at the LQT1 and LQT4 loci. No evidence for linkage was observed for the other LQT gene loci. Strong evidence for linkage to the QRS axis was found at LQT2 and LQT3, with some evidence for linkage at LQT4. Finally, some evidence for linkage to the T-wave excess was found for LQT2. We found no sex-related differences for the QTL effects. No evidence for linkage was observed for any of the other ECG parameters.

Discussion

We tested the hypothesis that LQT loci might be QTLs for the QTc in normal individuals and found that this was indeed the case for LQT1 and LQT4. We believe that these data are important because these respective genes could now be

TABLE 3. MZ-DZ Twin Analysis Showing Genetic and Environmental Effects

	Genetic Effect	Shared Environment	Nonshared Environment	χ^2/DF	*P*	r_{MZ}	r_{DZ}
RR		0.46	0.54	3.1/4	NS	0.50	0.37
P	0.46	0.12	0.42	9.6/3	0.01	0.55	0.40
PR		0.53	0.47	2.3/4	NS	0.47	0.51
QRS	0.40		0.60	7.2/4	0.01	0.41	0.08
QTc	0.52		0.48	1.3/4	0.01	0.52	0.30
P axis		0.23	0.77	2.8/4	NS	0.26	0.29
QRS axis	0.59		0.41	1.7/4	0.01	0.60	0.24
T axis	0.52		0.48	5.4/4	0.01	0.51	0.11

examined in detail for lesser allelic variants that might be functionally important. LQT syndromes are relatively rare, whereas rhythm disturbances in the general population are common. The discovery of QTLs in the normal population for QTc may elucidate causes for rhythm disturbances in the general population, allow the development of new diagnostic strategies, and enable the selection of individuals at increased risk.

Twin studies have been used previously to examine the effect of genetic variance on ECG parameters. Hanson et al[3] were able to study MZ and DZ twins reared apart and showed that PR interval, QRS duration, QRS axis, QTc, and ventricular rate indicated a significant contribution of genetic effects, ranging from 30% to 60%. Although their analysis is different from the analysis we used, the heritability estimates are similar. Hanson et al[3] were then able to compare data from twins reared apart and twins reared together and observed little difference in terms of ECG parameters. They provided firm evidence that genetic factors are of real importance in determining the basic physiological measures responsible for ECG components. Our MZ-DZ twin comparisons strongly support their conclusion. We were also able to confirm the finding that men have a shorter QTc interval than women of the same age.[6,17]

DZ twins are a particularly powerful sib-pair model because of identical ages and a shared environment, at least in childhood. Interestingly, a QTL for a closely defined reading disability has been described on chromosome 6, by means of sib-pair analysis including DZ twins.[18] In that study, the power of DZ twins in the sib-pair analysis was aptly demonstrated; DZ twin sib pairs exhibited a lod score twice that of nontwin affected siblings. This result would suggest that the sample size can be sharply reduced without a loss of power when DZ twin siblings are examined. The usefulness of DZ twins in the quantitative sib-pair linkage analysis approach to genes relevant to cardiovascular disease was recently demonstrated by Austin et al,[19] who found linkage between the microsomal triglyceride-transfer protein gene locus and plasma triglyceride concentrations, and also by Knoblauch et al,[20] who found linkage between the macrophage scavenger receptor gene locus and HDL-cholesterol concentrations. In previous studies, we found linkage between the *ACE* gene locus[5] and the *IGF-1* gene locus[21] and echocardiographically determined parameters of heart size in these same twin subjects.

TABLE 4. Sib-Pair Linkage Analysis in DZ Subjects

Locus	QTc	P Wave	QRS	QRS Axis	T Axis
LQT1	<0.001	0.99	0.06	0.99	0.99
LQT2	0.76	0.99	0.99	<0.001	0.004
LQT3	0.17	0.99	0.43	0.003	0.99
LQT4	<0.001	0.99	0.25	0.05	0.42
LQT5	0.99	0.17	0.06	0.99	0.55

P values for a QTL effect are given.

Congenital LQT is an autosomal-dominant genetic disorder of cardiac electrical repolarization caused by mutations of ≥6 genes.[2] Four LQT genes have been identified: *KVLQT1*, *HERG*, and *Min K* encode for cardiac potassium channels, whereas *SCN5A* encodes for the cardiac sodium channel. Altered ion-channel function produces prolongation of the action potential and propensity to torsade de pointes ventricular tachycardia. A fifth gene locus has been shown on chromosome 4; however, the gene has not yet been cloned.[22] The discovery of linkage to this locus in normal individuals could conceivably be useful in narrowing the region containing the responsible gene. The entire genomic structure of 3 LQT genes, including *KVLQT1*, has been described, allowing genetic screening to identify individuals at risk for this disorder.[23] Shimizu and Antzelevitch[24] examined the cellular basis for the ECG features of LQT1. They used a specific blocker of the I_{Ks} channel and prolonged the QT interval and action potential duration in an in vitro model. Our data would suggest that I_{Ks} channel activity is heritable, perhaps via variation in *KVLQT1*. After puberty, women with LQT are at greater risk for arrhythmias than men.[25] In our analysis, we found no sex-specific genetic effects on linkage with LQT loci; however, our numbers may not have been sufficient to identify a difference.

We were surprised to find strong evidence for linkage between the QRS vector and LQT2, as well as LQT3, whereas neither LQT2 or LQT3 was linked to QTc. The first locus contains *HERG*, whereas the second contains *SCN5A*. We are not aware of specific QRS-axis aberrations in LQT patients except while they experience polymorphic ventricular tachycardia.[26] El-Sherif et al[27] recently observed a localized circuit that varied its location and orientation from beat to beat, which serves to explain the transition of the QRS axis during polymorphic ventricular tachycardia in LQT. The

presence of LQT ion channels in the conduction system might be consistent with genetic linkage with certain channel loci and the QRS axis normally. We observed a significant genetic effect on the QRS axis in the MZ-DZ twin comparison. The *HERG* locus was also linked to the T-wave axis. Phenotypic T-wave patterns are often abnormal in LQT .[28]

The phenotype of LQT varies depending on the specific mutation involved.[29] Phenotypic heterogeneity is also caused by variable penetrance and expressivity. We believe that our identification of the *KVLQT1* locus as a QTL for QTc and 2 other LQT loci as QTLs for the QRS axis in normal, healthy individuals has direct clinical implications. For instance, the structure of *KVLQT1*, *HERG*, *SCN5A*, and the gene on chromosome 4, when it is cloned, will enable a strategy of multiplex sequencing in these individuals and their parents.[30] Allelic variants having a functional bearing on QTc or QRS axis can be identified in these healthy persons, which can then be tested in patients with congestive heart failure at risk for developing cardiac arrhythmias. For example, an allelic variant in the β_2-adrenergic receptor gene, which strongly influences survival in heart failure patients, was recently described.[31] Prospective strategies to influence QTc, thereby avoiding cardiac arrhythmias, could then be applied.[32]

Acknowledgment

This study was supported by a grant-in-aid to Dr Luft by the Bundesministerium für Bildung und Forschung.

References

1. McDonagh TA, Morrison CE, Lawrence A, Ford I, Tunstall-Pedoe H, McMurray JJV, Dargie HJ. Symptomatic and asymptomatic left-ventricular systolic dysfunction in an urban population. *Lancet*. 1997;350: 829 833.
2. Vincent GM. The molecular genetics of the long QT syndrome: genes causing fainting and sudden death. *Annu Rev Med*. 1998;49:263 274.
3. Hanson B, Tuna N, Bouchard T, Heston L, Eckert E, Lykken D, Segal N, Rich S. Genetic factors in the electrocardiogram and heart rate of twins reared apart and together. *Am J Cardiol*. 1989;63:606 609.
4. Busjahn A, Faulhaber H-D, Viken RJ, Rose RJ, Luft FC. Genetic influences on blood pressure with the cold pressor test: a twin study. *J Hypertens*. 1996;14:1195 1199.
5. Busjahn A, Knoblauch J, Knoblauch M, Bohlender J, Menz M, Faulhaber H-D, Becker A, Schuster H, Luft FC. Angiotensin converting enzyme and angiotensinogen gene polymorphisms, plasma levels, and left ventricular size: a twin study. *Hypertension*. 1997;29:165 170.
6. Bazett HC. An analysis of the time relationship of electrocardiograms. *Heart*. 1920;7:353 370.
7. Martin N, Boomsma D, Machin G. A twin-pronged attack on complex traits. *Nat Genet*. 1997;17:387 392.
8. Becker A, Busjahn A, Faulhaber H-D, Bähring S, Schuster H, Luft FC. Automated zygosity determination with microsatellites. *J Reprod Med*. 1997;42:260 266.
9. Wu D-A, Bu X, Warden CH, Shen DDC, Jeng C-Y, Sheu WHH, Fuh MMT, Katsuya T, Dzau VJ, Reaven GM, Lusis AJ, Rotter JI, Chen Y-DI. Quantitative trait locus mapping of human blood pressure to a genetic region at or near the lipoprotein lipase gene locus on chromosome 8p22. *J Clin Invest*. 1996;97:2111 2118.
10. Eaves LJ, Neale MC, Maes H. Multivariate multipoint linkage analysis of quantitative trait loci. *Behav Genet*. 1996;26:519 525.
11. Neale MC. *Mx: Statistical Modeling*. 4th ed. Medical College of Virginia, Richmond, Va: Department of Psychiatry; 1997.
12. Kruglyak L, Lander ES. Complete multipoint sib-pair analysis of qualitative and quantitative traits. *Am J Hum Genet*. 1995;57:439 454.
13. *S.A.G.E: Statistical Analysis for Genetic Epidemiology* [computer program]. Release 2.2. Cleveland, Ohio: Department of Epidemiology and Biostatistics, Case Western Reserve University; 1996.
14. Fulker DW, Cherny SS. An improved multipoint sib-pair analysis of quantitative traits. *Behav Genet*. 1996;26:527 532.
15. Lander ES, Kruglyak L. Genetic dissection of complex traits: guidelines for interpreting and reporting linkage results. *Nat Genet*. 1995;11: 241 246.
16. Neale MC, Cardon LR. *Methodology for Genetic Studies of Twins and Families*. Dordrecht, Netherlands: Kluwer Academic Publishers; 1992:496.
17. Ebert SN, Liu XK, Woosley RL. Female gender as a risk factor for drug-induced cardiac arrhythmias: evaluation of clinical and experimental evidence. *J Womens Health*. 1998;7:547 557.
18. Cardon LR, Smith SD, Fulker DW, Kimberling WJ, Pennington BF, DeFries JC. Quantitative trait locus for reading disability on chromosome 6. *Science*. 1994;266:276 279.
19. Austin MA, Talmud PJ, Luong L-A, Haddad L, Day INM, Newman B, Edwards LK, Krauss RM, Humphries SE. Candidate-gene studies of the atherogenic lipoprotein phenotype: a sib-pair linkage analysis of DZ women twins. *Am J Hum Genet*. 1998;62:406 419.
20. Knoblauch H, Busjahn A, Münter S, Nagy Z, Faulhaber H-D, Schuster H, Luft FC. Heritability analysis of lipids and three gene loci in twins link the macrophage scavenger receptor to high-density lipoprotein cholesterol concentrations. *Arterioscler Thromb Vasc Biol*. 1997;17:2054 2060.
21. Nagy Z, Busjahn A, Bähring S, Faulhaber H-D, Gohlke H-R, Knoblauch H, Rosenthal M, Müller-Myhsok B, Schuster H, Luft FC. Quantitative trait loci for blood pressure exist near the IGF-1, the Liddle syndrome, the angiotensin II-receptor gene, and the renin loci in man. *J Am Soc Nephrol*. 1999. In press.
22. Schott JJ, Charpentier F, Peltier S, Foley P, Drouin E, Bouhour JB, Donnelly P, Vergnaud G, Bachner L, Moisan JP. Mapping of a gene for long QT syndrome to chromosome 4q25 27. *Am J Hum Genet*. 1995;57: 1114 1122.
23. Splawski I, Shen J, Timothy KW, Vincent GM, Lehmann MH, Keating MT. Genomic structure of three long QT syndrome genes: KVLQT1, HERG, and KCNE1. *Genomics*. 1998;51:86 97.
24. Shimizu W, Antzelevitch C. Cellular basis for the ECG features of the LQT1 form of the long-QT syndrome. *Circulation*. 1998;98:2314 2322.
25. Locati EH, Zareba W, Moss AJ, Schwartz PJ, Vincent GM, Lehmann MH, Towbin JA, Priori SG, Napolitano C, Robinson JL, Andrews M, Timothy K, Hall WH. Age- and sex-related differences in clinical manifestations in patients with congenital long-QT syndrome: findings from the International LQTS Registry. *Circulation*. 1998;97:2237 2244.
26. Roden DM, Lazzara R, Rosen M, Schwartz PJ, Towbin J, Vincent GM, for the SADS Foundation Task Force on LQTS. Multiple mechanisms in the long-QT syndrome. *Circulation*. 1996;94:1996 2012.
27. El-Sherif N, Chinushi M, Caref EB, Restivo M. Electrophysiological mechanism of the characteristic electrocardiographic morphology of torsade de pointes tachyarrhythmias in the long-QT syndrome. *Circulation*. 1997;96:4392 4399.
28. Moss AJ, Zareba W, Benhorin J, Locati EH, Hall WJ, Robinson JL, Schwartz PJ, Towbin JA, Vincent GM, Lehmann MH, Keating MT, MacCluer JW, Timothy KW. ECG T-wave patterns in genetically distinct forms of the hereditary long QT syndrome. *Circulation*. 1995;92: 2929 2934.
29. Zareba W, Moss AJ, Schwartz PJ, Vincent GM, Robinson JL, Prior SG, Benhorin J, Locati EH, Towbin JA, Keating MT, Lehmann MH, Hall WJ. Influence of genotype on the clinical course of the long-QT syndrome: International Long-QT Syndrome Registry Research Group. *N Engl J Med*. 1998;339:960 965.
30. Wendel B, Hoehe MR. The human mu opioid receptor gene: 5′ regulatory and intronic sequences. *J Mol Med*. 1998;76:461 463.
31. Liggett SB, Wagoner LE, Craft LL, Hornung RW, Hoit BD, McIntosh TC, Walsh RA. The ILe 164 β2-adrenergic receptor polymorphism adversely affects the outcome of congestive heart failure. *J Clin Invest*. 1998;102:1534 1539.
32. Choy AM, Lang CC, Chomsky DM, Rayos GH, Wilson JR, Roden DM. Normalization of acquired QT prolongation in humans by intravenous potassium. *Circulation*. 1997;96:2149 2154.

Genetic and Environmental Influences on Coping Styles: A Twin Study

ANDREAS BUSJAHN, PHD, HANS-DIETER FAULHABER, MD, KRISTINA FREIER, MD AND FRIEDRICH C. LUFT, MD

Objective: Coping styles are generally considered to be environmentally driven, primarily by family influences. However, because personality traits are commonly influenced by genetic effects, we hypothesized that heredity is also important for coping. **Methods:** We tested this hypothesis by assessing 19 coping styles, as well as four secondary coping factors, by questionnaire in 212 pairs of monozygotic and dizygotic twins. We then examined heredity by structural equation modeling. **Results:** All coping styles showed evidence of genetic influences. The coping styles shared one common genetic factor. In addition, each coping style was also influenced by other separate genetic factors. Shared environment had no significant influence on coping styles. Three of 19 more specific coping styles showed shared environmental effects as well as genetic influences, 14 were solely under genetic influences, and two showed only shared environment effects. **Conclusions:** We suggest that hereditary effects on certain coping style preferences cannot be explained solely by genetic influences on major personality traits and temperament. An analysis of the relationships between coping and personality in twin subjects may elucidate the distinction between genetic and environmental effects. **Key words:** coping, genetics, environment, twins.

DZ = dizygotic; MZ = monozygotic; SD = standard deviation; SVF = Stressverarbeitungs-Fragebogen (coping questionnaire).

INTRODUCTION

The psychological processes aimed at diminishing or terminating stress are called "coping" processes (1). A working definition of coping might be "the things people do to avoid being harmed by life-strains" (2). Complex factors, such as personality, attitudinal, cognitive, and expectancy elements, are involved (3). Coping is generally assumed to be a learned behavior; however, genetic factors also play a role (4). Kendler et al. (5) studied female twins and used the 14-item "Ways of Coping Checklist." They identified three scales, turning toward others, problem solving, and denial. Their heritability estimates were 30% for turning toward others and problem solving; however, they could find no significant shared environmental influence on these two coping styles. On the other hand, the influence of shared environment for denial was significant at 20%, but no genetic effect was found. Kendler et al. also showed that the equal environment assumption was valid for the coping behaviors measured. Neither the number of social contacts between the twins nor environmental similarities in childhood influenced the test scores. These results agree with those from a study in children aged 9 to 16 years (6). Mellins et al. (6) assessed coping with a structured interview and questionnaire. Four of seven coping scales exhibited genetic influences in their study, two were influenced by shared environmental factors, and one was influenced by both. Another twin study examining genetic influences on coping styles was performed in the context of life situation and self-reported health in Sweden (7). Although heritability was not estimated, the intraclass correlations for MZ and DZ twins in that study allow a rough estimate. The questionnaire used gives a single result for the "sense of coherence," with MZ correlations more than twice those of same-sex DZ correlations. This result indicates heritability due to nonadditive genetic influences.

Additional indirect evidence can be drawn from studies relating coping to personality. There are well-established relationships between major personality traits, like neuroticism or extroversion, and different coping styles, with about 20% of coping variance being explained by personality (see Ref. 8 for review). Because personality itself is partially heritable, such influences may indirectly affect coping as well. However, neither coping nor personality is static. Thus, the correlation between the two is not necessarily unidirectional; coping might influence personality development in adulthood as well as in childhood (9). To gain insight into the genetic influences on coping, we performed a study in adult male and female MZ and DZ twins to test the hypothesis that coping styles are influenced by genetic variability. We then tested the hypothesis that different coping styles share some genetic influences and also have other unique, unshared genetic components.

METHODS

Subjects

Our sample consisted of 212 twin pairs, 117 MZ twins (81 female and 36 male) and 95 DZ twins (47 female, 12 male, and 36 different sexes), aged 34 ± 14 years, recruited by calls for volunteers in articles about twin research published in the print media (10–12).

From the Franz Volhard Clinic, Max Delbrück Center for Molecular Medicine, Medical Faculty of the Charité, Humboldt University, Berlin, Germany.

Address reprint requests to: Andreas Busjahn, PhD, Franz Volhard Clinic, Wiltbergstr. 50, 13122 Berlin, Germany. Email: busjahn@fvk-berlin.de

Received for publication November 17, 1998; revision received March 31, 1999.

0033-3174/99/6104-0469

MZ and female twins exceeded the expected population mean, a common phenomenon in self-selected twin samples. Because the ages, gender distribution, and geographic origins within Germany were not significantly different between the zygosity groups, and because sex-specific heritability was not estimated, the results of our study are likely to be unbiased. The sample was not selected with respect to any of the phenotypes measured. The protocol was approved by the Ethics Committee on Human Subjects of Humboldt University, and written informed consent was obtained from each subject. Zygosity determinations were made with five microsatellite markers coamplified by polymerase chain reaction (13). The probability of a same-sex DZ twin pair being concordant for all markers tested was .006.

Measures

Coping was assessed with use of the German SVF questionnaire (14). The questionnaire was completed by the twins under the supervision of a trained research nurse. The questionnaire includes 19 scales for different types of reactions to an unspecified range of situations that impair, adversely affect, irritate, or disturb the emotional equilibrium or balance of the subject. The questionnaire is similar to the dispositional form of the COPE questionnaire (15). Each scale consists of six items that are answered using a five-point scale according to the probability of that reaction. Example items of the SVF are listed in Table 1. The reliability of all scales was sufficiently high (median Cronbach's α = 0.8; see Table 2 for test–retest reliability reported by the test authors). The validity has been tested by intercorrelations between subscales, by correlations with a variety of questionnaires, and by specification of different stressful situations. Correlations between SVF scales and neuroticism ranged from 0.00 to 0.59, and correlations between SVF scales and extroversion ranged from 0.00 to 0.36, confirming the relationship between coping and personality described by Watson and Hubbard (8). Normal values (T scale; mean = 50 and SD = 10) for the SVF for German volunteers according to gender for the 20- to 64-years-old age range are available. No age effects were found in the standardized sample.

Because the subtests of the SVF questionnaire were not totally independent, secondary factors based on the intercorrelation were determined by the test authors and confirmed in our own sample. In

TABLE 2. SVF Questionnaire Scores by Zygosity and Reliability Coefficients (Test–Retest Correlation After 4 weeks, n = 200), given by the test authors for the standardization sample (19)[a]

SVF Scale	MZ Twins (Mean ± SD)	DZ Twins (Mean ± SD)	Test–Retest Reliability
Play down	49 ± 10	48 ± 9	0.72
Compare with others	51 ± 10	51 ± 10	0.84
Guilt defense	52 ± 9	52 ± 8	0.77
Distraction from situation	48 ± 8	49 ± 9	0.72
Substitutional satisfaction	52 ± 9	54 ± 10	0.80
Ego boost	52 ± 9	52 ± 9	0.80
Situational control	49 ± 9	51 ± 9	0.69
Reaction control	46 ± 9	47 ± 9	0.77
Positive self-instruction	47 ± 9	48 ± 9	0.78
Need for social support	55 ± 8	54 ± 7	0.80
Avoidance	49 ± 8	49 ± 10	0.77
Flight tendency	50 ± 10	50 ± 10	0.70
Social retreat	50 ± 9	49 ± 10	0.79
Rumination	50 ± 9	49 ± 9	0.82
Resignation	50 ± 9	48 ± 9	0.82
Self-pity	49 ± 9	48 ± 9	0.82
Self-accusation	51 ± 9	50 ± 9	0.68
Aggression	52 ± 8	52 ± 10	0.84
Self-medication/alcohol use	47 ± 8	47 ± 7	0.86

[a] A score of 50 is the mean score for normal German men and women.

TABLE 1. The German SVF Questionnaire (19)[a]

Scale	Coping Mechanism	Response
All questions start with the statement "When I have been upset by anybody, disturbed by anything, or somehow thrown off balance, . . ."		
1	Play down	I tell myself, "It is not that bad."
2	Compare with others	I tell myself, "Others couldn't take it the way I do."
3	Guilt defense	I tell myself, "I'm not to blame."
4	Distraction from situation	I try to concentrate on something else.
5	Substitutional satisfaction	I treat myself by buying something nice.
6	Ego boost	I think about my success in other situations.
7	Situational control	I make a plan how to solve the problem.
8	Reaction control	I try to keep my behavior under control.
9	Positive self-instruction	I tell myself not to give up.
10	Need for social support	I try to talk to someone about the problem.
11	Avoidance	I start to avoid this kind of situation.
12	Flight tendency	I only want to get out of this.
13	Social retreat	I prefer to be by myself.
14	Rumination	I think about it over and over.
15	Resignation	I tend to give up.
16	Self-pity	I ask myself, "Why me?"
17	Self-accusation	I tell myself, "After all, it's my fault."
18	Aggression	I get enraged.
19	Self-medication/alcohol use	I'll have a few beers.

[a] Example items of the coping questionnaire are given. Items are rated on a scale of 1 to 5.

a four-factor solution, 61% of variance is explained; in a six-factor solution, 72% is explained. Two of the six factors are very specific and combine just two scales. We therefore elected to reduce the number of coping factors in relation to sample size and based our analysis on the four-factor solution. The four factors can be characterized as follows: SVF1, defense (play down, compare with others, guilt defense, and ego boost); SVF2, emotional coping (flight, social retreat, rumination, resignation, self-accusation, and aggression); SVF3, substitution (substitutional satisfaction, need for social support, and self medication/alcohol abuse); and SVF4, active coping (situational control, reaction control, positive self-instruction, and avoidance).

Statistical Analyses

Statistical analyses were performed using a computer program (SPSS Inc., Chicago, IL). Heritability estimates were obtained by structural equation modeling using the Mx computer program (16, 17). By comparing MZ twin variances and covariances with those of DZ twins, we estimated the relative influence of additive genetic factors (a^2), nonadditive genetic factors (d^2), shared environmental factors (c^2), and unique environmental factors (e^2) contributing to individual differences in coping behavior. Additive genetic variance is the genetic variance associated with the average effect of alleles without allelic interaction or gene–gene interaction. Dominance (allelic interaction within a gene) or epistasis (gene–gene interaction) will lead to nonadditive genetic variance. Shared environmental factors are those operating within families and provide a potential source of family resemblance, as opposed to unique (or random) environmental factors, which contribute to differences between family members. Strong additive genetic effects will lead to significant correlations in MZ and DZ twins, with the MZ correlation twice the DZ correlation. Nonadditive genetic effects or dominance will lead to an increase in the difference between MZ and DZ twin correlations, because the chance of a DZ twin pair sharing two alleles identical by descent is only 25%. Interactions between gene loci (epistasis) will affect the difference in the MZ/DZ twin correlation in the same way but with an even stronger effect when more than two loci are involved. Epistasis is not included in the model but will increase estimates of dominance. Epistasis in complex phenotypes is highly important; however, estimating epistasis is extremely difficult (18). Another source of strong nonadditive genetic variance is the dependency of gene action on the genetic background, as was shown in plant genetics (19). Shared environmental influences will increase MZ and DZ correlations to the same extent, thus decreasing the difference between correlations. The shared environment consists of the family environment and shared influences of school, social class, etc., whereas the nonshared environment includes factors that are unique for each member of a twin pair. Measurement errors will contribute to this source of variation as well; these errors cannot be corrected within sibling pairs. Although nonadditive genetic effects and shared environment might influence a given trait, they are confounded. Nonadditive genetic effects and shared environment have opposite effects on DZ covariance. Thus, the two effects cannot be estimated simultaneously in the same model. We began with a model encompassing additive genetic influences (A), shared influences (C), and nonshared environmental influences (ACE model) and fitted nested models without genetic and/or shared environmental influences. If the MZ correlation of a coping scale was more than twice the DZ correlation, nonadditive genetic effects (D) were tested by fitting ADE and nested models as a post hoc test. The sum of additive and nonadditive genetic influences in an ADE model equals the heredity as estimated in an ACE model. Thus, our analysis does not overestimate heritability. Full and nested models were compared by likelihood ratio χ^2 tests, models with the same *df* by the Akaike's information criterion, as described elsewhere (17, 20). Testing multiple measures within one sample increases the probability of type 2 errors. Thus, heritability estimates for the questionnaire's 19 subscales and the four secondary factors are given to illustrate results, to allow comparison with other, more specific coping scales, and to provide starting points for the generation of future hypotheses.

We performed a multivariate path analysis to test for genetic influences on coping. This approach extends the univariate heritability analysis by allowing genetic covariance to be shared by different traits. Thus, the correlation between phenotypes is tested for underlying common genetic factors. An example for the bivariate case with no shared environmental influences is given in Figure 1. Factor Ac is a genetic influence shared by both phenotypes, whereas As is a genetic factor specific to phenotype 2. Corresponding environmental influences are Ec and Es. This bivariate case can be extended to multiple measures. By Cholesky decomposition, a phenotypic variance/covariance matrix is factored as the product of a triangular matrix and its transposition. By comparing nested models, we tested whether all four coping factors shared genetic influences as well as the related hypothesis that all genetic influences on the different coping styles were attributable to a single shared genetic factor. This approach permitted the completion of submodels with a single genetic factor and four unshared genetic factors.

RESULTS

Scores for the SVF questionnaire subscales are given in Table 2. None of the scales showed a significant difference between MZ and DZ twins nor a significant deviation from the expected population mean of 50. Thus, our MZ and DZ twins seem to be fairly representative of the German population. Because the scores are gender specific, the overrepresentation of female twins will not influence the results. Table 3 shows the twin analysis and provides the significant effects of additive genetic factors (a^2), nonadditive genetic factors (d^2), shared environmental factors (c^2), and nonshared environmental factors (e^2). There was

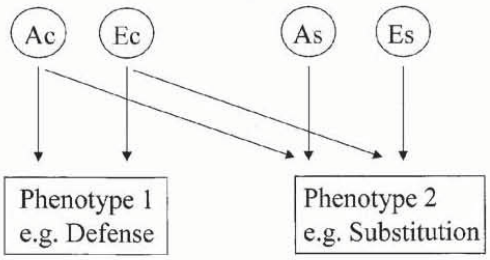


Fig. 1. Bivariate path model that decomposes the phenotypic variance and covariance for two given phenotypes, for example, two different coping styles, into additive genetic (Ac) and environmental (Ec) contributions that are common to both phenotypes and factors that are specific to one phenotype only, that is, additive genetic (As) and environmental (Es) contributions.

TABLE 3. Percentage of Total Variance Explained by Additive Genetic Factors (a^2), Nonadditive Genetic Factors (d^2), Shared Environmental Factors (c^2), and Nonshared Environmental Factors (e^2). Together With the Fit Index (c^2/df) for the Selected Model[a]

SVF Scale	a^2	d^2	c^2	e^2	c^2/df	rMZ/rDZ
Defense (factor 1)		0.52		0.48	6.8/4	0.47/0.25
Emotional coping (factor 2)		0.23		0.77	4.4/4	0.29/0.04
Substitution (factor 3)		0.41		0.59	3.9/4	0.42/−0.09
Active coping (factor 4)	0.21		0.09	0.70	2.3/3	0.32/0.17
Play down		0.23		0.77	5.4/4	0.26/−0.08
Compare with others		0.43		0.57	1.6/4	0.44/−0.02
Guilt defense		0.68		0.32	5.5/4	0.36/−0.11
Distraction from situation			0.06	0.94	2.2/4	0.14/0.14
Substitutional satisfaction	0.10	0.12		0.78	3.2/3	0.21/0.09
Ego boost		0.28		0.72	1.3/4	0.29/−0.02
Situational control	0.26		0.13	0.61	3.2/3	0.41/0.25
Reaction control		0.22		0.78	2.8/4	0.23/0.00
Positive self-instruction	0.06	0.30		0.64	3.6/4	0.37/0.10
Need for social support		0.35		0.65	5.0/4	0.36/0.09
Avoidance	0.27		0.07	0.66	6.7/3	0.29/0.24
Flight tendency		0.48		0.52	1.6/4	0.49/0.17
Social retreat		0.38		0.62	1.8/4	0.35/0.16
Rumination	0.43			0.57	2.2/4	0.43/0.20
Resignation	0.34	0.20		0.46	1.8/3	0.53/0.23
Self-pity	0.24		0.14	0.62	1.6/3	0.37/0.27
Self-accusation		0.35		0.65	4.7/4	0.36/−0.01
Aggression			0.39	0.61	4.1/4	0.38/0.41
Self-medication/alcohol use	0.23	0.21		0.56	1.6/3	0.45/0.16

[a] Only significant ($p < .05$) data are presented. In addition, the phenotypic correlation r of MZ and DZ is given. Presented are the four coping factors we derived for our reductionist model, followed by the 19 questionnaire items.

no homogeneous pattern of heritability, although numerous parameters showed genetic variability. Fourteen of the 19 scales showed genetic influences without significant shared environmental effects. On the other hand, two scales showed effects of family environment without genetic effects. Finally, three scales showed a combination of genetic and shared environmental effects.

Table 4 gives the correlations within and between twin pairs for secondary coping factors. These relationships form the basis of the subsequent analysis. The results of the multivariate path analysis are given in Table 5, which shows the parameter estimates for the best-fitting model. We were interested in the hypothesis that coping styles are not only influenced by specific genetic factors but also share some genetic influences. We had no specific hypotheses as to which coping scales might share genetic influences. Therefore, we did not further reduce the model. The corresponding fit indices are presented in Table 6 for the fully saturated model (I); the selected model without shared environmental effects, which allowed genetic variance and covariance (II); a reduced model allowing only specific genetic variance for each of the four coping factors (III); and a model with only one genetic factor shared by all coping factors (IV). Dropping the shared environmental influences from the saturated model did not affect model fit, which is in agreement with the univariate analysis of the secondary coping factors. The model allowing only four specific genetic factors without overlap between coping styles (model III) resulted in a significant decrease in fit. When the model was tested with only one common genetic factor without additional specific genetic influences (model IV), the fit decreased significantly as well. Thus, the results support a complex genetic architecture with specific as well as shared genetic influences for each coping behavior.

DISCUSSION

The important finding in our study is that the four coping factors, defense, emotional coping, substitution, and active coping, showed evidence of genetic variance. These factors were derived from a 19-scale coping style questionnaire; 17 of the 19 coping styles showed evidence of heritability. We demonstrated that there is no single genetic factor shared by all different coping styles but rather that there are both specific and shared genetic influences for different coping factors. In terms of these secondary factors, there was no evidence of influences of shared environment on coping. However, this finding does not allow the conclusion that shared environmental influences on coping are

TABLE 4. Between-Twin and Within-Twin Correlations for Secondary Coping Factors Derived From the SVF Questionnaire for MZ and DZ Twins[a]

	Twin 1				Twin 2			
	SVF1	SVF2	SVF3	SVF4	SVF1	SVF2	SVF3	SVF4
MZ, Twin 1								
SVF1	1.00							
SVF2	0.03	1.00						
SVF3	0.32	0.35	1.00					
SVF4	0.36	0.22	0.29	1.00				
MZ, Twin 2								
SVF1	0.35	−0.04	−0.11	0.20	1.00			
SVF2	−0.15	0.47	0.12	−0.11	−0.14	1.00		
SVF3	0.11	0.23	0.32	−0.05	0.16	0.47	1.00	
SVF4	0.10	−0.08	0.04	0.30	0.41	0.02	0.23	1.00
DZ, Twin 1								
SVF1	1.00							
SVF2	−0.11	1.00						
SVF3	0.20	0.37	1.00					
SVF4	0.34	0.20	0.12	1.00				
DZ, Twin 2								
SVF1	−0.15	0.05	−0.17	−0.13	1.00			
SVF2	−0.16	0.27	0.22	−0.02	−0.06	1.00		
SVF3	−0.20	0.02	0.10	−0.24	0.25	0.31	1.00	
SVF4	−0.20	0.00	−0.05	0.04	0.40	−0.03	−0.04	1.00

[a] Secondary coping factors are as follows: SVF1 = defense; SVF2 = emotional coping; SVF3 = substitution; SVF4 = active coping.

TABLE 5. Genetic and Environmental Variances for the Selected Model II (No Shared Environmental Effects)[a]

	SVF1	SVF2	SVF3	SVF4
Genetic factors				
1	0.27			
2	−0.11	0.48		
3	−0.06	0.17	0.30	
4	0.09	−0.10	−0.08	0.28
Environmental factors				
1	0.73			
2	0.04	0.52		
3	0.29	0.21	0.70	
4	0.29	0.20	0.25	0.72

[a] Genetic variance (heritability) is on the upper diagonal; covariances are off diagonal. Numbers represent the amount of genetic influences (top) and environmental influences (bottom) shared by the four coping factors derived from the German SVF questionnaire: SVF1 = defense; SVF2 = emotional coping; SVF3 = substitution; SVF4 = active coping.

TABLE 6. Model Fit Statistics for Multivariate Models[a]

Model	c^2/df	p	Change in c^2/df	p
I. Saturated ACE	54.4/42	0.10		
II. No common environment	55.5/52	0.35	1.1/10	NS[b]
III. Only specific genetic factors	76.3/58	0.06	20.8/6	.01[c]
IV. Only 1 common genetic factor	127.1/58	0.00	71.6/6	.01[c]

[a] Additive (A) genetic, shared (C), and nonshared environmental (E) influences comprise model I, the ACE model. Model II is without shared environmental effects. Model III allows only specific genetic variances for each of the four SVF coping factors. Model IV allows for one common genetic variance to be shared by all the SVF coping factors.
[b] NS = not significant versus model I.
[c] Versus model II.

negligible. Shared environment could exert effects on a different level, such as on more specific coping strategies. To allow generation of more specific hypotheses on genetic and environmental influences, we performed structural equation modeling for all 19 scales of the coping questionnaire. We understand that the comparison of so many phenotypes with a moderate sample size raises problems of multiple testing and can only illustrate the issue without allowing for statistical testing of hypotheses regarding differences between coping scales.

As in earlier studies, we did not find a homogeneous heritability pattern. Seventeen of the 19 coping scales showed some evidence of genetic variance. Eight showed evidence of additive genetic factors, and 13 were suggestive of nonadditive genetic effects. These nonadditive genetic influences might indicate interactions between genes in the complex genetic background of psychological traits. Such nonadditive genetic influences have been found in other studies

(10–12) and may be systematic in twin studies on personality traits, as confirmed in larger samples (21). Shared environmental factors were important for five coping styles, whereas all 19 involved nonshared environmental factors. The moderate 0.5 correlations in MZ twins emphasize the importance of individual environmental influences and possible gene–environment interactions. Given the good reliability estimates for the coping questionnaire, measurement errors should have only a minor influence. Although the preference of coping strategies is influenced by genetic factors, individual coping strategies are developed in cointeraction with the environment.

The first three coping styles (play down, compare with others, and guilt defense) can all be classified as intrapsychic behavior and form an internal dialogue. Nevertheless, we cannot generally conclude that internal coping behavior is heritable and that external coping behavior is learned. Scale 4, distraction from situation, is a cognitive strategy as well but nevertheless showed no genetic influence. The next coping style, substitutional satisfaction, is an acting-out behavior, although the motivation for this kind of behavior in parents would not necessarily be clear to their children. The genetic component of this behavior with the lack of shared environmental influences would argue against learning from parental example in the development of this behavior.

Situational control is accepted as an appropriate and mature way of handling problems and is likely to be consciously included by parents as a strategy in child rearing. Shared environment is likely to complement genetic influences and reflects upbringing as well as learning by example. The reaction control mechanism is an internal coping style defined by the lack of external reactions to stress. This mechanism is less likely to be learned by mimicking the parents, as is reflected by the lack of shared environmental influences in the presence of genetic effects. Scales 11 and 12, avoidance and flight tendency, are both related to the trait of fear; however, they showed differences in family transmission. Although the feeling of fear and thereby the wish to leave threatening situations has a genetic component, the ability to control the related behavior (avoidance) is also influenced by the family environment. A rather surprising result was revealed for the aggression scale (scale 18). We found no genetic influence for this variable, even though in a personality questionnaire we obtained a significant heritability score of 55% for the aggression trait (A. Busjahn, unpublished observations). A possible explanation could be that stress-related reactive aggression is independent of personality and may instead be learned from parental models. The genetic influence found for self-medication/alcohol use is in agreement with studies on alcohol and drug abuse and indicates that this strategy at least partially serves as stress relief.

Our results suggest that heritable and learned coping styles cannot be discriminated by means of a simplistic classification such as inner versus outer behavior. Furthermore, there was no clear pattern in the correlations between coping styles, with and without genetic influences, and either neuroticism or extroversion (19). The genetic factors relevant for coping may be the same factors influencing major personality traits. Given the correlation between major personality traits and coping styles, which ranged from 0.00 to 0.60 (19), and given the complex genetic architecture of coping, only a small part of coping heritability is likely to be due to the inheritance of personality traits. Although this hypothesis is only speculative, multivariate analyses of both coping and personality in extended samples may give better insight into this complex relationship.

Interindividual differences in coping behavior patterns are best explained by simultaneous environmental and genetic interactions. Because the structuring of the questionnaire is only one of many possibilities, different types of classification might result in different heritability patterns. Although our hypothesis of genetic influences on coping was confirmed, the extent of genetic influences can be only roughly estimated from our results. However, our data highlight the necessity of considering genetic influences in this field. We believe the twin subjects were representative of the German population, because the scale results were not significantly different between MZ and DZ twins. Furthermore, the mean population score of 50 was closely approximated by MZ and DZ twins in all scale categories.

We believe our results extend beyond the study of personality traits. Coping strategies are accompanied by simultaneous adjustments in autonomic nervous system tone, heart rate, heart rate variability, baroreceptor function, and blood pressure. These physiological variables may have a bearing on chronic cardiovascular disease. A significant impact of stress responses on cardiovascular risk has been demonstrated (22, 23). To better understand the relationship between stressors and physiological responses, the mediating factors of personality and coping warrant investigation (24–26). Furthermore, improved molecular genetic techniques combined with twin studies will allow assessment of candidate genes influencing physiological (27) and psychological behavior (28). The DZ twins and their parents lend themselves to linkage analysis with molecular genetic markers, after which additional association studies are possible on

the same sample. We used this approach in earlier studies (29).

In summary, our data in a large number of normal male and female twins confirm a strong genetic influence on coping strategies. We found that most (17 of 19) coping styles were under the influence of genetic variability. Both additive and nonadditive genetic effects were observed. Our data suggest that a simplistic notion of inner (genetic) and outer (learned) behaviors must be revised. Each individual behavior is a complex interplay of heredity and environment. Given the fact that psychological variables show striking similarities across countries (30), we believe that our results apply to countries outside Germany.

We thank Professor Alois Angleitner and his colleagues at the University of Bielefeld for their support in recruiting participants.

REFERENCES

1. Costa PT, McCrae RR. Personality, stress, and coping: some lessons from a decade of research. In: Markides KS, Cooper CL, editors. Aging, stress, and health. New York: John Wiley and Sons; 1989. p. 269–85.
2. Pearlin LI, Schooler C. The structure of coping. J Health Soc Behav 1978;19:2–21.
3. Feifel H, Strack S, Nagy VT. Coping strategies and associated features of medically ill patients. Psychosom Med 1987;49: 616–25.
4. Theorell T. Life events and manifestations of ischemic heart disease: epidemiological and psychological aspects. Psychosomatics 1980;34:135–48.
5. Kendler KS, Kessler RC, Heath AC, Neale MC, Eaves LJ. Coping: a genetic epidemiological investigation. Psychol Med 1991;21: 337–46.
6. Mellins CA, Gatz M, Baker L. Children's methods of coping with stress: a twin study of genetic and environmental influences. J Child Psychol Psychiatry 1996;37:721–30.
7. Lange AL, Fischbein S. Life situation, self-reported health and coping ability of 35-year-old twins and controls: a follow-up of a longitudinal Swedish twin study at adolescence. Acta Genet Med Gemellol (Roma) 1996;45:405–16.
8. Watson D, Hubbard B. Adaptational style and dispositional structure: coping in the context of the five-factor model. J Pers 1996;64:737–74.
9. Aldwin CM, Sutton KJ, Lachmann M. The development of coping in adulthood. J Pers 1996;64:837–72.
10. Ball D, Hill L, Freeman B, Eley TC, Strelau J, Riemann R, Spinath FM, Angleitner A, Plomin R. The serotonin transporter gene and peer-rated neuroticism. Neuroreport 1997;8:1301–4.
11. Busjahn A, Faulhaber H-D, Viken RJ, Rose RJ, Luft FC. Genetic influences on blood pressure with the cold pressor test: a twin study. J Hypertens 1996;14:1195–9.
12. Busjahn A, Knoblauch J, Knoblauch M, Schuster H, Bohlender J, Faulhaber H-D, Luft FC. Angiotensin converting enzyme and angiotensinogen gene polymorphisms, plasma levels, and left ventricular size: a twin study. Hypertension 1997;29:165–70.
13. Becker A, Busjahn A, Faulhaber HD, Baehring S, Robertson J, Schuster H, Luft FC. Twin zygosity: automated determination with microsatellites. J Reprod Med 1997;42:260–6.
14. Jahnke W, Erdmann G, Kallus W. Der Stressverarbeitungs-Fragebogen [The coping questionnaire]. Göttingen: Verlag für Psychologie; 1985.
15. Carver CS, Scheier MF, Weintraub JK. Assessing coping strategies: a theoretically based approach. J Pers Soc Psychol 1989;56:267–83.
16. Neale MC. Mx: Statistical modeling, 4th ed. Richmond (VA): Department of Psychiatry, University of Virginia; 1997.
17. Neale MC, Cardon LR. Methodology for genetic studies of twins and families. Dordrecht: Kluwer; 1992.
18. Frankel WN, Schork NJ. Who's afraid of epistasis? Nat Genet 1996;14:371–3.
19. Lynch M, Walsh B. Genetics and analysis of quantitative traits. Sunderland, UK: Sinauer; 1997.
20. Akaike H. Factor analysis and AIC. Psychometrika 1987;52: 317–32.
21. Eaves LJ, Heath AC, Neale MC, Hewitt JK, Martin NG. Sex differences and nonadditivity in the effects of genes on personality. Twin Res 1998;1:131–7.
22. Alderman MH, Ooi WL, Madhavan S, Cohen H. Blood pressure reactivity predicts myocardial infarction among treated hypertensives. J Clin Epidemiol 1989;43:859–66.
23. Rostrup M, Westheim A, Kjeldsen SE, Eide I. Cardiovascular reactivity, coronary risk factors, and sympathetic activity in young men. Hypertension 1993;22:891–9.
24. Müller MM, Elbert T. Ärgerverarbeitung bei kardiovascular Erkrankten [Coping with anger by cardiovascular patients]. Psychother Psychosom Med Psychol 1994;44:240–6.
25. Perini C, Smith DH, Neutel JM, Smith MA, Henry JP, Buhler FR, Weiner H, Weber MA. A repressive coping style protecting from emotional distress in low-renin essential hypertensives. J Hypertens 1994;12:601–7.
26. Vogele C, Steptoe A. Emotional coping and tonic blood pressure as determinants of cardiovascular responses to mental stress. J Hypertens 1992;10:1079–87.
27. Knoblauch H, Busjahn A, Münter S, Nagy Z, Faulhaber H-D, Schuster H, Luft FC. Heritability analysis of lipids and three gene loci in twins link the macrophage scavenger receptor to high-density lipoprotein cholesterol concentrations. Arterioscler Thromb Vasc Biol 1997;17:2054–60.
28. Plomin R, Caspi A. DNA and personality. Eur J Pers 1998;12: 387–407.
29. Nagy Z, Busjahn A, Bähring S, Faulhaber H-D, Gohlke H-R, Knoblauch H, Schuster H, Luft FC. Quantitative trait loci for blood pressure exist near the IGF-1, the Liddle syndrome, and the angiotensin II-receptor gene loci in man. J Am Soc Nephrol. In press 1999.
30. Weissman MM, Bland RC, Canino GJ, Faravelli C, Greenwald S, Hwu HG, Joyce PR, Karam EG, Lee CK, Lellouch J, Lepine JP, Newman SC, Rubio-Stipec M, Wells JE, Wickramaratne PJ, Wittchen H, Yeh EK. Cross-national epidemiology of major depression and bipolar disorder. JAMA 1996;276:293–9.

Peroxisome Proliferator–Activated Receptor γ Gene Locus Is Related to Body Mass Index and Lipid Values in Healthy Nonobese Subjects

Hans Knoblauch, Andreas Busjahn, Bertram Müller-Myhsok, Hans-Dieter Faulhaber, Herbert Schuster, Regina Uhlmann, Friedrich C. Luft

Abstract—The peroxisome proliferator–activated receptor γ (*PPARγ*) gene has been implicated in morbid obesity and is important to lipid and carbohydrate metabolism. However, the relevance of gene variations in healthy nonobese subjects has not been defined. We recruited monozygotic and dizygotic healthy nonobese twin subjects to test the hypothesis that the *PPARγ* gene is important to body mass index and lipid concentrations in healthy nonobese subjects. Both linkage and association strategies were used in the same dizygotic twins. The *PPARγ* gene locus was linked ($P<0.01$) to high-density lipoprotein cholesterol, low-density lipoprotein cholesterol, and body mass index as quantitative traits. A biallelic variant in the *PPARγ* gene was associated with high-density lipoprotein cholesterol and body mass index ($P<0.05$). We also looked for linkage between the same variables and the retinoic X receptor gene locus. This locus was linked to total and low-density lipoprotein cholesterol as well as triglycerides. We conclude that the *PPARγ* gene is highly relevant to lipid metabolism and body mass index, not only in the morbidly obese but also in healthy nonobese subjects. The same appears to be true for its binding partner. Sequencing these genes in twins would serve to identify gene variations contributing to body mass index and lipid concentrations in healthy nonobese subjects. (***Arterioscler Thromb Vasc Biol.* 1999;19:2940-2944.)**

Key Words: genetics ■ *PPARγ* ■ quantitative trait loci ■ body mass index ■ cholesterol, HDL ■ cholesterol, LDL ■ twins

Peroxisome proliferator–activated receptor γ (PPARγ) is a member of the nuclear hormone receptor superfamily that heterodimerizes with the retinoid X receptor and functions as a transcriptional regulator of genes linked to lipid metabolism and energy balance. The thiazolidine class of antidiabetic drugs and 15-deoxy-Δ12,14-prostaglandin J2 are ligands for this receptor.[1–3] PPARγ expression is highest in adipose tissue but is detectable at lower levels in other tissues (eg, kidney and liver).[4,5] Tontonoz et al[6] recently reported that PPARγ promotes monocyte/macrophage differentiation and uptake of oxidized LDL cholesterol. Ristow et al[7] recently reported a *PPARγ* mutation in 4 patients with massive obesity. When expressed in fibroblasts, the mutated gene accelerated lipid uptake of the cells and caused them to differentiate into adipocytes. These observations prompted us to test the hypothesis that the *PPARγ* locus is linked to lipid values and body mass index (BMI) in healthy nonobese dizygotic (DZ) twin subjects in terms of a quantitative trait locus (QTL). We then took advantage of a biallelic marker in the *PPARγ* gene and were able to associate the genotypes with BMI and HDL cholesterol. We also searched for linkage between the retinoic X receptor gene locus and the same phenotypes and showed that this locus is a QTL for total and LDL cholesterol. Our data support the notion that PPARγ and its binding partner are relevant to BMI and lipid levels in healthy nonobese persons.

Methods

Study Population

We recruited 222 pairs of monozygotic (MZ; 122 pairs) and DZ (100 pairs) twins by advertisement to participate in studies involving blood pressure regulation and cardiovascular phenotypes.[8,9] We also recruited the parents of the DZ twins to permit identity by descent linkage analysis. Twin zygosity was verified with use of 5 polymerase chain reaction–amplified microsatellite markers as described in detail elsewhere.[10] The subjects were all healthy, normotensive whites recruited from various parts of Germany. The protocol was approved by the university's committee on the protection of human subjects, and written informed consent was obtained from all participants. Persons with histories of familial lipid disorders were excluded.

Phenotypic Characterization

Blood was obtained from the twins after a 12-hour fast. Total cholesterol, HDL cholesterol, and triglycerides were determined by automated methods.[11] LDL cholesterol concentrations were calcu-

Received March 8, 1999; revision accepted April 9, 1999.

From the Franz Volhard Clinic and Max Delbrück Center for Molecular Medicine (H.K., A.B., H.-D.F., H.S., R.U., F.C.L.), Charité, Medical Faculty of the Humboldt University of Berlin, Berlin; and Bernhard Nocht Institute for Tropical Medicine (B.M.-M.), Department of Molecular Medicine, Hamburg, Germany.

Correspondence to Friedrich C. Luft, Franz Volhard Clinic, Wiltbergstrasse 50, 13122 Berlin, Germany. E-mail: luft@fvk-berlin.de

Arterioscler Thromb Vasc Biol. **is available at http://www.atvbaha.org**

TABLE 1. Clinical Data and Serum Lipid Values

Variable	MZ	DZ
Pairs, n	122	100
Age, y	34±15	34±13
Sex (male/female), n	80/164	60/140
Height, cm	169±9	170±9
Weight, kg	67±13	71±14
BMI, kg/m^2	23±4	24±4
Total cholesterol, mg/dL*	183±39	193±42
HDL cholesterol, mg/dL*	51±14	57±17
LDL cholesterol, mg/dL*	115±34	115±32
Triglycerides, mg/dL†	87±67	100±64

Values are mean±SD.

*To convert cholesterol values into millimolar concentration (mmol/L), multiply the given value (mg/dL) by 0.01129.

†To convert triglyceride values into millimolar concentration (mmol/L), multiply the given value (mg/dL) by 0.0286.

lated by the Friedewald equation.[12] Blood was also obtained for determination of zygosity and other molecular genetic studies.

Genotyping

Microsatellite markers spanning ≈45 cM around the *PPARγ* gene on chromosome 3[13] (D3S1297, D3S1304, D3S3726, D3S3589, D3S1263, D3S3608, D3S2338, and D3S1266), as well as markers spanning 5 cM around the retinoic X receptor gene on chromosome 1 (D1S2768, D1S2844, D1S426, and D1S194), were analyzed using the PE Applied Biosystems genotyping system. A polymorphism corresponding to a silent C-to-T substitution in exon 6 of *PPARγ* was analyzed according to a published protocol.[14]

Statistical Analysis

For linkage analysis, only DZ pairs and their parents were included. Data were analyzed by using a structural equation modeling (SEM) approach[15] as implemented in the Mx statistical package.[16] This approach is based on variance-covariance matrices of sibs weighted by the probability of sharing 0, 1, or 2 alleles identical by descent. The higher power of the variance-covariance–based analysis, compared with the squared trait differences–based approach by the Haseman-Elston regression method,[17] was shown in a recent simulation study.[18] Because we used a candidate gene approach, we accepted $P<0.01$ to test for significant linkage in accordance with the criteria defined by Lander and Kruglyak.[19] To increase the power for the association analysis, mean scores of pairs of MZ twins were included together with scores of DZ pairs.[20] Statistical analysis was conducted by using ANOVA (SPSS).

Results

Table 1 shows the demographic and lipid-related variables for the twin subjects. Female sex was twice as common as male sex. Subjects were young adults of normal height, weight, and BMI. Total, HDL, and LDL cholesterol and triglyceride values were all within normal limits. Table 2 shows the results of the heritability analysis. A major genetic effect was demonstrated on all lipid parameters, although strong environmental effects were also shown. Table 3 gives the results of the structural equation modeling multipoint linkage analysis for markers spanning the *PPARγ* and retinoic X receptor gene loci and body size as well as for serum lipid concentrations. Only the peak value for each locus is given. Significant linkage was found between the *PPARγ* locus and BMI, body weight, and serum HDL and LDL cholesterol levels. For the retinoic X receptor locus, linkage was found for total and LDL cholesterol levels, as well as triglycerides. Figure 1 shows the results of the linkage analysis for *PPARγ* and HDL cholesterol. The location of the markers and the *PPARγ* gene is indicated in the figure. BMI and LDL cholesterol reached their peak significance in the same chromosomal region as HDL cholesterol. Table 4 gives the results of an association analysis using the biallelic marker in the *PPARγ* gene. Persons with the TT variant had decidedly higher HDL cholesterol values, as shown in Figure 2, and tended to have lower LDL cholesterol values and the lowest BMI compared with persons with the CC or CT variant.

TABLE 2. Genetic and Environmental Effects on Serum Lipid Values

	Genetic Effect	Nonshared Environment	c^2/DF	P	r_{MZ}	r_{DZ}
Total cholesterol	0.64	0.36	1.0/4	0.01	0.65	0.37
HDL cholesterol	0.59	0.41	0.4/4	0.01	0.63	0.30
LDL cholesterol	0.66	0.34	0.7/4	0.01	0.66	0.36
Triglycerides	0.72	0.28	0.6/4	0.01	0.72	0.44
Body weight	0.89	0.11	1.4/4	0.01	0.96	0.46
BMI	0.97	0.03	0.65/4	0.01	0.85	0.46

Fit indices for the reported models and P values for the genetic effect are given together with correlation coefficients for MZ and DZ.

We next examined within-pair differences in MZ twins, biallelic marker–concordant DZ twins, and biallelic marker–discordant DZ twins. For HDL cholesterol, MZ twins had the least pair difference, DZ concordant twins were intermediate, and DZ discordant twins had the greatest within-pair HDL cholesterol concentration difference ($P<0.01$). These results are shown in Figure 3. Similar results were obtained for LDL cholesterol (data not shown). For BMI, the results of the analysis were not significant.

TABLE 3. Results of Linkage Analysis

	PPARγ		RXR	
	c^2 Model Difference	P (SEM)	c^2 Model Difference	P (SEM)
Total cholesterol	1.8	0.18	4.35	0.001
HDL cholesterol	11.0	0.0009	0	NS
LDL cholesterol	7.8	0.005	6.23	0.004
Triglycerides	2.0	0.15	12.5	0.0004
Body weight	10.0	0.0015	0	NS
BMI	7.4	0.005	0	NS

Difference between SEM models with and without a QTL effect and corresponding P values for a QTL effect are given.

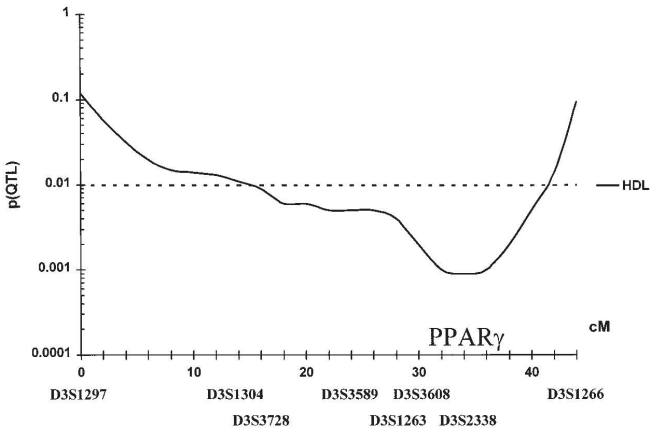


Figure 1. Results of multipoint linkage analysis for HDL cholesterol and markers at the *PPARγ* gene locus. The approximate location of the gene is indicated. The line represents the error value p for the existence of a QTL. A threshold of $P=0.01$ was set. The lowest p value over the *PPARγ* locus is indicated in Table 3.

Discussion

Because cardiovascular disease is the most common cause of death worldwide, more than half of the population can expect to develop cardiovascular disease during their lifetime.[21] Thus, identifying QTL for cardiovascular disease–relevant phenotypes in healthy nonobese persons, and subsequently demonstrating variations in the corresponding candidate genes, is important to cardiovascular disease genetics. We believe that our study exemplifies the utility of this combined linkage and association approach. MZ and DZ twins permit quantification of genetic and environmental variance. As reported earlier,[22] the body size parameters and serum lipid concentrations were all influenced by genetic variance. DZ twins, who are perfectly matched for age and who are generally exposed to very similar environmental conditions, are ideal subjects for efficient sib-pair analyses. Thus, far fewer subjects are needed to gain insight into QTL.

We showed that the *PPARγ* gene locus is a QTL for BMI, LDL, and HDL cholesterol concentrations in healthy nonobese subjects and that a biallelic polymorphism in the *PPARγ* gene is associated with BMI and lipid concentrations. The relationship between LDL cholesterol and *PPARγ*, as well as the relationship between HDL cholesterol and *PPARγ* achieved significance, whereas that for total cholesterol and *PPARγ* did not. We interpret this finding as suggesting that the effects on LDL and HDL cholesterol may be opposite in nature. Our association results would support that point of view. Furthermore, the retinoic X receptor gene locus is a QTL for LDL and total cholesterol. Our results are in concordance with data from a total genome scan conducted in 92 nuclear families using several measures of obesity.[23] In this study, a marker on chromosome 3 (D3S1286) was significantly linked to the percentage of body fat. This marker is located within the chromosomal region linked to BMI in our study. A second genomic scan conducted in Pima Indians, which pointed toward a QTL for BMI on chromosome 11, showed no significant linkage.[24] That lack of confirmation may have been due to population specifics or power restrictions. *PPARγ* has been shown to be an important disease gene for morbid obesity.[7] In a segregation analysis, evidence was found for at least 2 major loci influencing BMI.[25] Together, these loci are expected to account for 64% of the variance in BMI.

Although obesity is common, most persons do not have morbid obesity. Our data suggest that more subtle variations in *PPARγ* are important to BMI and lipid values in healthy nonobese subjects as well. We believe that these observations are particularly relevant for several reasons. Obesity is reported as the most common health problem in developed countries.[26] Low HDL cholesterol values are a recognized risk for coronary heart disease.[27] PPARγ is pivotal to a variety of serious obesity-related medical conditions, including type 2 diabetes mellitus and cardiovascular disease. Although adipose tissue has been recognized as a principal site of *PPARγ* gene expression, the gene is expressed at lower levels in many nonadipose tissues and cell types, where it

TABLE 4. Results of Association Analysis

	CC	CT	TT	*P*
Total cholesterol, mg/dL*	176.5±35.1	187.2±30.9	185.2±26.4	0.176
HDL cholesterol, mg/dL*	49.5±13.1	50.1±11.9	64.2±13.0	0.001
LDL cholesterol, mg/dL*	110.3±30.9	122.6±30.3	104.9±27.4	0.061
Triglycerides, mg/dL†	84.1±54.3	74.1±36.3	80.7±38.6	0.544
BMI, kg/m^2	23.6±3.9	25.3±5.1	22.6±2.4	0.026
Body weight, kg	68.1±12.7	69.6±14.8	66.8±14.5	0.665

*To convert cholesterol values into millimolar concentration (mmol/L), multiply the given value (mg/dL) by 0.01129.

†To convert cholesterol values into millimolar concentration (mmol/L), multiply the given value (mg/dL) by 0.0286.

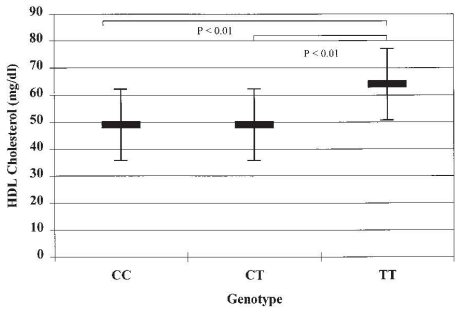


Figure 2. Results of the association analysis for the *PPARγ* polymorphism in exon 6 and HDL cholesterol. The data suggest that the T allele exerts a recessive action.

may also play an important role. Several classes of ligands have been found.[28–31] The thiazolidinediones are specific synthetic agonists for *PPARγ*. 15-deoxy-Δ12,14-prostaglandin J2 is a natural ligand. Certain polyunsaturated fatty acids, such as linoleic acid, also activate PPARγ. Nonsteroidal antiinflammatory drugs, such as ibuprofen, can activate the receptor as well. PPARγ also functions as an obligate heterodimer with the retinoic X receptor, which, among other things, is involved in triglyceride metabolism.[32]

PPARγ may actively participate in the pathogenesis of atherosclerosis. Monocytes and macrophages are pivotal to inflammation and the development of arteriosclerosis. Ricote et al[33] were able to show that PPARγ is markedly upregulated in activated macrophages. They found that PPARγ inhibits the expression of inducible nitric oxide synthase, gelatinase B, and the scavenger receptor A genes in response to synthetic ligands, probably by antagonizing the transcription factors AT-1, STAT, and NF-κB. Tontonoz et al[5] found that PPARγ is induced in human monocytes after exposure to oxidized LDL and is expressed at high levels in atherosclerotic lesions. Ligand activation of PPARγ induced monocyte differentiation and promoted the transcriptional induction of the scavenger receptor. Nagy et al[34] further elucidated this issue by showing that oxidized LDL components acted as endogenous PPARγ ligands. They demonstrated a novel signaling pathway coordinated by the macrophage scavenger receptor on the cell surface internalizing the particle and PPARγ in the nucleus, which is transcriptionally activated by its component lipids. Thus, PPARγ appears to be a key regulator of foam cell gene expression.

Our data suggest that *PPARγ* gene variants in healthy nonobese, nonhyperlipidemic subjects may have significant influence on BMI and plasma lipids. Such variants may have a great effect on the propensity to obesity, type 2 diabetes, and cardiovascular disease in later life. We can only speculate on the interrelationships among BMI, HDL cholesterol concentrations, and PPARγ. However, in epidemiological studies, a higher BMI is associated with lower HDL cholesterol concentrations,[35] consistent with our findings. Furthermore, Meirhaeghe et al[14] have described an interaction between the C-to-T substitution in the *PPARγ* gene and BMI for plasma leptin levels. They found that persons bearing at least one T allele had a lower BMI for a given leptin level, compared with CC homozygous individuals. Their results are consistent with our findings.

Deeb et al[36] recently demonstrated that the Pro12/Ala substitution in the *PPARγ* gene is associated with lower BMI and improved insulin sensitivity. The investigators were also able to show that the Pro12/Ala substitution is associated with decreased receptor activity. Yen et al[37] first reported this missense *PPARγ* mutation, which involves a C-to-G substitution at nucleotide 34. They also found an association between this mutation and type 2 diabetes mellitus in a small number of white patients. Ringel et al[38] were unable to confirm these findings in a large association study involving 522 type 1 diabetic and 503 type 2 diabetic patients, compared with 310 nondiabetic control subjects. Discrepancies in association studies are common. We believe our twin model may be more stable because we relied on both linkage and association approaches. Furthermore, our DZ sib pairs have half of their genes in common and therefore provide a much more homogeneous sample. For example, if ≈30 genes are responsible for obesity, the DZ twin would have concordant alleles for half of these genes. If we then examine discor-

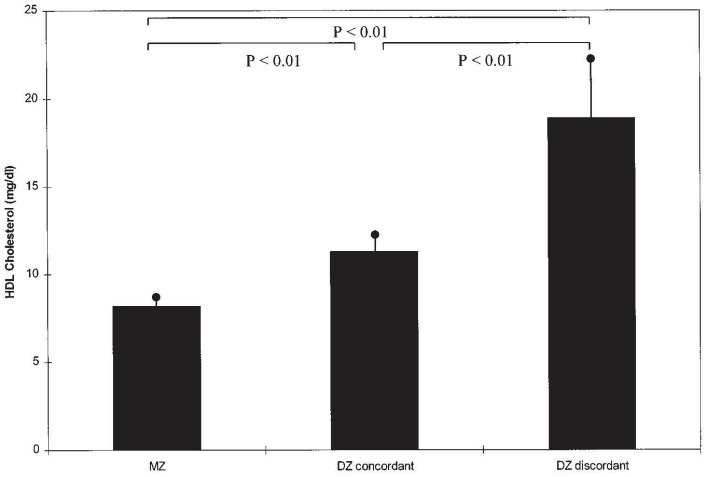


Figure 3. Within-pair differences in MZ twins and DZ twins concordant and discordant for the biallelic polymorphism in exon 6 of the *PPARγ* gene. The difference between concordant DZ and MZ twins indicates that in addition to *PPARγ*, other genes are also responsible for HDL cholesterol values.

dancy in a candidate gene, the number of confounders in our study would be decidedly less compared with that in association studies in randomly selected people.

The variant we examined is silent but apparently in linkage disequilibrium with a functional polymorphism in the *PPARγ* gene or possibly in a nearby gene. We suggest that much of the genetic variance on BMI and HDL cholesterol levels in healthy nonobese persons is attributable to the *PPARγ* gene locus. Multiplex sequencing of the *PPARγ* gene in all of our DZ twin subjects and their parents may provide insight into the functional variants involved. These findings highlight the broad, encompassing role of *PPARγ* in processes involving BMI and lipid metabolism not only in persons with disease but also in healthy, healthy nonobese persons.

References

1. Forman BM, Umensono K, Chen J, Evans RM. Unique response pathways are established by allosteric interactions among nuclear receptors. *Cell*. 1995;81:541 550.
2. Kliewer SA, Umensono K, Mangelsdorf FJ, Evans RM. Retinoid X receptor interacts with nuclear receptors in retinoic acid, thyroid hormone, and vitamin D3 signaling. *Nature*. 1992;355:446 449.
3. Lehmann JM, Moore LB, Smith-Oliver TA, Wilkison WO, Wilson TM, Kliewer SA. An antidiabetic thiazolidinedione is a high affinity ligand for peroxisome proliferator-activated receptor γ (PPARγ). *J Biol Chem*. 1995;270:12953 12956.
4. Dreyer C, Krey G, Keller H, Givel F, Helftenbein G, Wahli W. Control of the peroxisomal β-oxidation pathway by a novel family of nuclear hormone receptors. *Cell*. 1992;68:879 887.
5. Tontonoz P, Hu E, Spiegelman BM. Stimulation of adipogenesis in fibroblasts by PPAR gamma 2, a lipid-activated transcription factor. *Cell*. 1994;79:1147 1156.
6. Tontonoz P, Nagy L, Alvarez JGA, Thomazy VA, Evans RM. PPARγ promotes monocyte/macrophage differentiation and uptake of oxidized LDL. *Cell*. 1998;93:241 252.
7. Ristow M, Müller-Wieland D, Pfeiffer A, Krone W, Kahn CR. Obesity associated with a mutation in a genetic regulator of adipocyte differentiation. *N Engl J Med*. 1998;339:953 959.
8. Busjahn A, Faulhaber H-D, Viken RJ, Rose RJ, Luft FC. Genetic influences on blood pressure with the cold pressor test: a twin study. *J Hypertens*. 1996;14:1195 1199.
9. Busjahn A, Knoblauch H, Knoblauch M, Schuster H, Bohlender J, Faulhaber H-D, Luft FC. Angiotensin converting enzyme and angiotensinogen gene polymorphisms, plasma levels, and left ventricular size: a twin study. *Hypertension*. 1997;29:165 170.
10. Becker A, Busjahn A, Faulhaber H-D, Bähring S, Schuster H, Luft FC. Automated zygosity determination with microsatellites. *J Reprod Med*. 1997;42:260 266.
11. Allain CC, Poon LS, Chan CSG, Richmond W, Fu PC. Enzymatic determination of total serum cholesterol. *Clin Chem*. 1974;20:470 475.
12. Friedewald WT, Lewy RI, Fredrickson DS. Estimation of plasma low-density lipoprotein cholesterol concentration without use of the preparative ultracentrifuge. *Clin Chem*. 1972;18:499 501.
13. Beamer BA, Negri C, Yen CJ, Gavrilova O, Rumberger JM, Durcan MJ, Yarnall DP, Hawkins AL, Griffin CA, Burns DK, Roth J, Reitman M, Shuldiner AR. Chromosomal localization and partial genomic structure of the human peroxisome proliferator activated receptor gamma (hPPAR gamma) gene. *Biochem Biophys Res Commun*. 1997;233:756 759.
14. Meirhaeghe A, Fajas L, Helbecque N, Cottel D, Lebel P, Dallongeville J, Deeb S, Auwerx J, Amouyel P. A genetic polymorphism of the peroxisome proliferator activated receptor gamma gene influences plasma leptin levels in obese humans. *Hum Mol Genet*. 1998;7:435 440.
15. Eaves LJ, Neale MC, Maes H. Multivariate multipoint linkage analysis of quantitative trait loci. *Behav Genet*. 1996;26:519 525.
16. Neale MC. *Mx: Statistical Modeling*. 4th ed. Richmond, Va: Department of Psychiatry; 1997.
17. Kruglyak L, Lander ES. Complete multipoint sib-pair analysis of qualitative and quantitative traits. *Am J Hum Genet*. 1995;57:439 454.
18. Fulker DW, Cherny SS. An improved multipoint sib-pair analysis of quantitative traits. *Behav Genet*. 1996;26:527 532.
19. Lander ES, Kruglyak L. Genetic dissection of complex traits: guidelines for interpreting and reporting linkage results. *Nat Genet*. 1995;11: 241 246.
20. Miller MB. Monozygotic twins increase power of genetic association studies of complex phenotypes. *Behav Genet*. 1998;28:1024. Abstract.
21. Murray CJL, Lopez AD. Mortality by cause for eight regions of the world: Global Burden of Disease Study. *Lancet*. 1997;349:1269 1276.
22. Knoblauch H, Busjahn A, Münter S, Nagy Z, Faulhaber H-D, Schuster H, Luft FC. Heritability analysis of lipids and three gene loci in twins link the macrophage scavenger receptor to HDL cholesterol concentrations. *Arterioscler Thromb Vasc Biol*. 1997;17:2054 2060.
23. Lee JH, Reed DR, Li WD, Xu WZ, Joo EJ, Kilker RL, Nanthakumar E, North M, Sukul H, Bell C, Price RA. Genome scan for human obesity and linkage to markers in 20q13. *Am J Hum Genet*. 1999;64:196 209.
24. Hanson RL, Ehm MG, Pettitt DJ, Prochazka M, Thompson DB, Timberlake D, Foroud T, Kobes S, Baier L, Burns DK, Almasy L, Blangero J, Garvey WT, Bennett PH, Knowler WC. An autosomal genomic scan for loci linked to type II diabetes mellitus and body-mass index in Pima Indians. *Am J Hum Genet*. 1998;63:1130 1138.
25. Borecki IB, Blangero J, Rice T, Perusse L, Bouchard C, Rao DC. Evidence for at least two major loci influencing human fatness. *Am J Hum Genet*. 1998;63:831 838.
26. Rosenbaum M, Leibel RL, Hirsch J. Obesity. *N Engl J Med*. 1997;337: 396 407.
27. Criqui MH, Golomb BA. Epidemiologic aspects of lipid abnormalities. *Am J Med*. 1998;105:48S 57S.
28. Forman BM, Chen J, Evans RM. Hypolipidemic drugs, polyunsaturated fatty acids, and eicosanoids are ligands for peroxisome proliferator-activated receptors α and δ. *Proc Natl Acad Sci U S A*. 1997;94:4312 4317.
29. Kliewer SA, Sundseth SS, Jones SA, Brown PJ, Wisely GB, Koble CS, Devchand P, Wahli W, Willson TM, Lenhard JM, Lehmann JM. Fatty acids and eicosanoids regulate gene expression through direct interaction with peroxisome proliferator-activated receptors α and γ. *Proc Natl Acad Sci U S A*. 1997;94:4318 4323.
30. Krey G, Braissant O, LâHorset F, Kalkhoven E, Perroud M, Parker MG, Wahli W. Fatty acids, eicosanoids, and hypolipidemic agents identified as ligands of peroxisome proliferator-activated receptors by coactivator-dependent receptor ligand assay. *Endocrinology*. 1997;11:779 791.
31. Spiegelman BM. PPARγ in monocytes: less pain, any gain? *Cell*. 1998; 93:153 155.
32. Mukherjee R, Strasser J, Jow L, Hoener P, Paterniti JR, Heyman RA. RXR agonists activate PPARα-inducible genes, lower triglycerides, and raise HDL levels in vivo. *Arterioscler Thromb Vasc Biol*. 1998;18: 272 276.
33. Ricote M, Li AC, Willson TM, Kelly CJ, Glass CK. The peroxisome proliferator-activated receptor-γ is a negative regulator of macrophage activation. *Nature*. 1998;391:79 86.
34. Nagy L, Tontonoz P, Alvarez JGA, Chen HW, Evans RM. Oxidized LDL regulates macrophage gene expression through ligand activation of PPAR gamma. *Cell*. 1998;93:229 240.
35. Dwyer JT, Stone EJ, Yang M, Feldman H, Webber LS, Must A, Perry CL, Nader PR, Parcel GS. Predictors of overweight and overfatness in a multiethnic pediatric population: Child and Adolescent Trial for Cardiovascular Health Collaborative Research Group. *Am J Clin Nutr*. 1998;67: 602 610.
36. Deeb SS, Fajas L, Nemoto M, Pihlajamäki J, Mykkänen L, Kuusisto J, Laakso M, Fujimoto W, Auwerx J. A Pro12Ala substitution in PPARγ associated with decreased receptor activity, lower body mass index and improved insulin sensitivity. *Nat Genet*. 1998;20:284 287.
37. Yen CJ, Beamer BA, Negri C, Silver K, Brown KA, Yarnall DP, Burns DK, Roth J, Shuldiner AR. Molecular scanning of the human peroxisome proliferator activated receptor γ (hPPARγ) gene in diabetic Caucasians: identification of a Pro12Ala PPARγ 2 missense mutation. *Biochem Biophy Res Commun*. 1997;241:270 274.
38. Ringel J, Engeli S, Distler A, Sharma AM. Pro12Ala missense mutation of the peroxisome proliferator activated receptor γ and diabetes mellitus. *Biochem Biophys Res Commun*. 1999;254:450 453.

J Am Soc Nephrol 10: 1709–1716, 1999

Quantitative Trait Loci for Blood Pressure Exist Near the IGF-1, the Liddle Syndrome, the Angiotensin II-Receptor Gene and the Renin Loci in Man

ZSUZSANNA NAGY,* ANDREAS BUSJAHN,† SYLVIA BÄHRING,† HANS-DIETER FAULHABER,† HANS-RÜDIGER GOHLKE,† HANS KNOBLAUCH,† MAGDA ROSENTHAL,† BERTRAM MÜLLER-MYHSOK,‡ HERBERT SCHUSTER,† and FRIEDRICH C. LUFT†

**University of Pecs, Pecs, Hungary; †Franz Volhard Clinic and Max Delbrück Center for Molecular Medicine, Virchow Klinikum, Humboldt University of Berlin, and ‡Bernhard Nocht Institute, Hamburg, Germany.*

Abstract. Blood pressure (BP) is heritable and finding quantitative trait loci that influence BP is an important step in identifying genes responsible for BP regulation. Sixty-six pairs of dizygotic (DZ) twin subjects and their parents were used in a sib-pair analysis to look for linkage of selected candidate genes to the quantitative trait BP. Microsatellite markers were tested in the vicinity of the gene loci for insulin-like growth factor-1 (IGF-1), Liddle syndrome, autosomal-dominant hypertension with brachydactyly, angiotensinogen, angiotensin II type 1 receptor, angiotensin-converting enzyme, renin, and lipoprotein lipase. BP was measured in a standardized manner. Heart size was determined echocardiographically. Significant linkage was found at the IGF-1, Liddle syndrome, and AT_1 receptor gene for systolic BP. Linkage for diastolic BP was found at the autosomal-dominant hypertension with brachydactyly locus. Both systolic and diastolic BP were linked to the renin gene locus. The linkage was most consistent for the IGF-1 gene locus and systolic BP. Linkage was also found between the IGF-1 gene locus and posterior cardiac wall thickness, septal thickness, and left ventricular mass index. It is suggested that these quantitative trait loci may be important for the subsequent detection of allelic variants for elevated BP. Furthermore, these results linking the IGF-1 gene locus to both BP and cardiac dimensions underscore the importance of the IGF-1 gene as a candidate gene for cardiovascular disease.

Hypertension is a major public health problem, the treatment of which consumes a large portion of the health care budget (1). Clear evidence for familial transmission has existed for almost 80 yr (2), and results of twin and family studies have shown a substantial genetic component to the disorder, with heritable variation estimated at 30 to 60% (3). Some unusual forms of hypertension are inherited as a simple, monogenic trait; however, essential hypertension is complex and multiple genes are likely to be involved (4). BP itself is a complex variable, influenced by multiple interacting physiologic regulatory systems. Moreover, the diagnosis of hypertension is an arbitrary one. Mapping loci important for BP regulation would facilitate identification of the functional genes that potentially cause the disorder. Insight into as yet unappreciated mechanisms could result. We recruited monozygotic (MZ) and dizygotic (DZ) twins. The inclusion of the MZ twins enabled us to estimate heritability of BP for the study population. Furthermore, we performed a sib-pair analysis in the DZ twins, including genotypes of their parents, to look for loci related to the quantitative trait, BP. Such quantitative trait loci (QTL) could further the detection of allelic variants associated with elevated BP and thus be important in subsequent studies of hypertensive families or affected sib-pair analyses in hypertensive patients. We did not conduct a total genome scan in the DZ twins, but rather directed our attention to candidate gene loci.

The candidate gene loci we selected included components of the renin-angiotensin system. The renin gene itself has been identified as a QTL in the rat (5), hypertension (as a qualitative trait) has been linked to the angiotensinogen gene (6,7), and the angiotensin-converting enzyme (ACE) and angiotensin II type 1 receptor (AT_1) genes have been associated with increased cardiovascular risk (8). Liddle syndrome is a monogenic disorder (9) that closely resembles "low renin" hypertension. The condition is caused by hyperactivity of the epithelial amiloride-sensitive sodium channel in the distal renal tubule, leading to salt and volume retention. Autosomal-dominant hypertension with brachydactyly resembles essential hypertension. The responsible gene has been mapped to chromosome 12p (10); however, the gene has not yet been elucidated. The lipoprotein

Received November 30, 1998. Accepted February 19, 1999.

Zsuzsanna Nagy and Andreas Busjahn contributed equally to this work.

This work was presented in part at the American Society of Nephrology 30th annual meeting, Philadelphia, PA, 1998.

Correspondence to Dr. Friedrich C. Luft, Franz Volhard Clinic, Wiltberg Strasse 50, 13122 Berlin, Germany. Phone: +49 30 9417 2202; Fax: +49 30 9417 2206; E-mail: luft@fvk-berlin.de

1046-6673/1008-1709
Journal of the American Society of Nephrology

lipase gene locus has been identified as a susceptibility gene locus of hypertriglyceridemia (11), and may be relevant to so-called "familial dyslipidemic" hypertension (12). Insulin-like growth factor-1 (IGF-1) (somatomedin C) was found to be elevated in the plasma of hypertensive patients and was correlated with sodium/lithium countertransport, a transport system that is influenced by genetic variance (13). We concentrated our attention on BP and echocardiographically determined cardiac dimensions.

Materials and Methods

Patients

We recruited 166 pairs of twins (MZ 100 and DZ 66) by advertisement to participate in studies involving BP regulation and cardiovascular phenotypes (14,15). The subjects were all German Caucasians. They were recruited from various parts of Germany. The protocol was approved by the University's committee on the protection of human subjects, and written informed consent was obtained from all participants. Blood was obtained for the determination of zygosity and other molecular genetic studies from all the twins and the parents of the DZ twins. Each participant underwent a medical history and physical examination. None had hypertension or any other chronic medical illness. BP was measured after 5 min (two measurements, 1 min apart) with a standardized mercury sphygmomanometer in the sitting, standing, and recumbent position by a trained physician. The mean of the two measurements was used as the BP.

M-mode and two-dimensional echocardiograms were recorded with patients in the left-lateral decubitus position. M-mode tracings that were guided two-dimensionally were recorded from the short parasternal axis at the chordal level between the free edges of the mitral leaflets at the tips of the papillary muscles. Only tracings with optimal visualization of left ventricular interfaces were used. In our echocardiographic laboratory, the range of variability of observations by a single reader is 0 to 1.5 mm for the left ventricular dimensions and 0 to 0.5 mm for the wall thickness. Interventricular septal thickness and posterior-wall thickness were measured in all patients, and left ventricular dimensions were calculated by the Penn formula according to the guidelines of the American Society of Echocardiography (16).

For this linkage study, the DZ pairs were selected and used as ordinary sib pairs, but with the advantage of perfect age matching and reduced environmental variation affecting the phenotype. The power of the twin model in elucidation of complex genetic disease has recently been emphasized by Martin *et al.* (17). The MZ twins were used to estimate allele frequencies for the markers tested. The zygosity was verified with the use of five PCR-amplified microsatellite markers as described in detail elsewhere (18). We examined six microsatellite markers at the IGF-1 locus, three at the Liddle syndrome locus, four at the autosomal dominant hypertension with brachydactyly locus, three at the AT_1 and lipoprotein lipase locus, and two for the angiotensinogen, ACE, and renin loci, shown in Table 1.

We assessed linkage for BP as a continuous trait rather than differentiate between normotensive and hypertensive subjects (19). Sib-pair analysis to determine linkage does not require the specification of a genetic model. The underlying trait can follow either Mendelian or non-Mendelian modes of inheritance. Analysis was done by using a structural equation modeling (SEM) approach (20), as implemented in the MX-package (21). From the four alleles harbored by the parents for a given locus, each child randomly inherits two. Thus, a pair of sibs may have inherited either the same or different alleles. More specifically, they may share zero, one, or two alleles identical by descent (IBD). If the locus under study is a QTL, phenotypic similarity of sibs (measured by the covariance) should increase with the number of alleles they share. Assuming no dominance effects, the total variance of the trait is due to the genetic effect of the QTL (Var_{qtl}), remaining additive genetic effects (Var_{addGen}), and environmental influences (Var_{env}):

$$Var = Var_{qtl} + Var_{addGen} + Var_{env}$$

Accordingly, the covariance of the three types of sibs as determined by their IBD status can be predicted as follows:

$$Cov_{IBD0} = 0.5\ Var_{addGen}$$

$$Cov_{IBD1} = 0.5\ Var_{qtl} + 0.5\ Var_{addGen}$$

$$Cov_{IBD2} = Var_{qtl} + 0.5\ Var_{addGen}$$

For linkage analysis, a model is specified estimating Var_{qtl}, Var_{addGen}, and Var_{env} so that the likelihood of the empirical variance-covariance matrix of the sibs, weighted by the probability of sharing zero, one, or two alleles identical by descent, is maximized. For each sib pair and each locus, the proportion of alleles IBD, based on parental genotypes, is calculated using a multipoint approach as

Table 1. Marker information[a]

Locus	Marker	Heterozygosity Index
IGF-1	D12S332	0.66
chromosome 12	D12S346	0.88
	D12S58	0.80
	IGF-1	0.55
	D12S318	0.73
	D12S78	0.85
Liddle	D16S412	0.76
chromosome 16	D16S403	0.83
	D16S420	0.82
AT_1 receptor	D3S1555	0.73
chromosome 3	D3S1308	0.72
	D3S1299	0.72
LPL	D8S282	0.73
chromosome 8	D8S261	0.78
	LPL	0.80
ACE	GH	0.82
chromosome 17	D17S794	0.59
Hypertension/Brachydactyly gene	D12S310	0.48
chromosome 12	D12S1591	0.77
	D12S1688	0.74
	GATA 91-H01	0.71
Renin	D1S510	0.74
chromosome 1	D1S456	0.59
AGT	D1S251	0.83
chromosome 1	D1S459	0.79

[a] IGF-1, insulin-like growth factor-1; AT_1, angiotensin II type 1 receptor; LPL, lipoprotein lipase; ACE, angiotensin-converting enzyme; AGT, angiotensinogen.

implemented in MAPMAKER/SIBS (22). To test for a QTL effect, the difference in model fit for models with and without a QTL effect is calculated as a χ^2 statistic. Because we used a candidate gene approach, we accepted $P < 0.01$ to test for significant linkage in accordance with the criteria defined by Lander and Kruglyak (23).

Because the SEM method is very new, we also used the more established single-point Haseman-Elston approach as implemented in the SIBPAL program of the statistical analysis for genetic epidemiology (SAGE) package (24). To test for linkage, a linear regression analysis is carried out with the squared trait difference as the dependent variable and IBD as the independent variable. A power calculation is available for this test (25). To obtain an 80% power level to detect a QTL explaining 50% of total variance, 600 pairs are required. On the basis of simulated data, the power of the new SEM linkage analysis has been estimated in different settings for total heritability and QTL effect size (26). For a power level of 70% and a QTL effect size of 50%, 200 to 300 pairs are required depending on overall heritability. Sample sizes <100 pairs do allow only exclusion of QTL effects >70%. The high power of the variance-covariance-based analysis, nearly twofold compared with the squared trait differences-based approach by the Haseman-Elston method, has been confirmed in a recent simulation study (27). In conclusion, while significant linkage results obtained in smaller samples are still reliable, failure to detect linkage raises the issue of a lack of power and should not be interpreted as an exclusion.

Parameters of the quantitative genetic models were estimated by structural equation modeling using the MX program developed by Neale (21). The variability of any given phenotype within a population can be decomposed into genetic influences (Var_{addGen}), environmental influences shared by the twins within a family ($Var_{sharedEnv}$), and effects of random environment (Var_{env}):

$$Var = Var_{addGen} + Var_{sharedEnv} + Var_{env}$$

For MZ and DZ, the covariance of their phenotype is given by:

$$Cov_{MZ} = Var_{addGen} + Var_{sharedEnv}$$

$$Cov_{DZ} = 0.5\ Var_{addGen} + Var_{sharedEnv}$$

Statistical Analyses

Heritability analysis in twin studies can estimate additive components of genetic variability as well as two environmental influences, shared and nonshared (28). These values estimate the relative amount of the variable's influence on interindividual differences up to a sum of one. Genetic as well as environmental effects were estimated by the best fitting model as selected by the χ^2 value. Statistical analysis was conducted using the SPSS program. Adjustment of BP values for gender and age was done by multiple linear regression with the unstandardized residuals as the corrected phenotypes.

Results

Demographic data, BP values, and heredity estimates of BP and echocardiographic variables in 200 MZ and 132 DZ twins are given in Table 2. There were no significant differences between MZ and DZ twins for any of the variables examined. Systolic and diastolic BP were heritable. The heritability estimates for systolic BP were about double those for diastolic BP. The echocardiographic parameters also demonstrated strong evidence of heritability.

Table 3 shows the results of linkage analysis for the tested loci in the DZ twins using systolic and diastolic BP in the sitting, standing, and recumbent position as the phenotype. The BP values in the table are corrected for age and gender, although noncorrected values gave similar results. Markers at the IGF-1, Liddle syndrome, AT_1 receptor, and renin gene loci gave statistically significant evidence for linkage with systolic BP in the SEM analysis. Although maximum likelihood estimation of linkage in principle allows estimation of a putative QTL's size effect, together with its confidence interval, greater sample sizes are needed for these variance estimates. For example, the QTL effect of the IGF-1 locus on systolic BP is estimated at 68% of total variance, but with a 95% confidence interval between 22 and 85%. Thus, the current data allow only the conclusion of a QTL effect >0, which is nevertheless an important step. Using Haseman-Elston regression, the same loci were either significant or suggestive ($P < 0.05$) for systolic BP only in the sitting position. The ACE and lipoprotein lipase loci showed a significant relationship to only one of the BP measurements. Thus, the results are only suggestive and not definitive.

For diastolic BP, the locus for autosomal-dominant hypertension and brachydactyly and the renin gene were significant. Linkage for the markers at the IGF-1 locus and echocardiographically determined cardiac mass (Penn formula) was sig-

Table 2. Demographic data, phenotypic values (mean ± SD), heredity estimates (a^2), and correlations (r) for MZ and DZ[a]

Parameter	MZ Twins	DZ Twins	a^2 (r_{MZ}/r_{DZ})
n	200	132	
Age (yr)	29 ± 12	31 ± 12	
M/F	52/148	85/47	
Height (cm)	169 ± 8	170 ± 8	
Weight (kg)	65 ± 11	67 ± 12	
BMI (kg/m)	22.4 ± 3.5	22.8 ± 3.4	
Systolic BP sitting (mmHg)	118 ± 11	118 ± 10	0.81 (0.81/0.31)
Diastolic BP sitting (mmHg)	69 ± 9	71 ± 8	0.41 (0.80/0.59)
Posterior wall thickness (mm)	8.7 ± 1.6	8.6 ± 1.6	0.26 (0.48/0.26)
Septum (mm)	8.9 ± 1.7	8.8 ± 1.6	0.37 (0.64/0.37)

[a] MZ, monozygotic; DZ, dizygotic; BMI, body mass index.

Table 3. Linkage analysis for blood pressure (corrected for age and gender) in the sitting, standing, and recumbent position[a]

Locus	H/E	SEM					
	SBP Sitting	SBP Sitting	SBP Standing	SBP Recumbent	DBP Sitting	DBP Standing	DBP Recumbent
IGF-1 (chromosome 12)	0.00001	0.00005	0.0002	0.004	0.9	0.9	0.3
Liddle (chromosome 16)	0.012	0.026	0.010	0.9	0.9	0.9	0.9
AT_1 receptor (chromosome 3)	0.027	0.0008	0.32	0.0026	0.9	0.9	0.21
LPL (chromosome 8)	0.217	0.5	0.13	0.005	0.9	0.9	0.061
ACE (chromosome 17)	0.162	0.58	0.36	0.051	0.37	0.89	0.9
Hypertension/Brachydactyly (chromosome 12)	0.088	0.9	0.9	0.9	0.0012	0.024	0.0024
Renin (chromosome 1)	0.046	0.0009	0.000001	0.0018	0.0036	0.009	0.008
AGT (chromosome 1)	0.228	0.9	0.9	0.9	0.038	0.9	0.9

[a] For systolic blood pressure results of both H/E analysis and SEM analysis are given. H/E, Haseman-Elston; SEM, structural equation modeling; SBP, systolic BP; DBP, diastolic BP. Other abbreviations as in Table 1.

nificant ($P = 0.008$). For posterior wall and septum thickness, the results were highly suggestive ($P = 0.017$ and 0.012, respectively). This relationship remained significant even after correcting for possible influences of BP on cardiac dimensions. Using Haseman-Elston regression, a microsatellite within the IGF-1 gene gave evidence of linkage with the phenotype posterior wall thickness phenotype ($P = 0.008$). Figure 1 shows the relationship between the squared co-twin difference for systolic BP and proportion of alleles identical by descent in the DZ twins at the marker *D12S58* (Haseman-Elston approach). The relationship was highly statistically significant. Figure 2 shows the results of SEM analysis for cardiac mass and the markers at the IGF-1 gene locus. The χ^2 value indicates a model fit difference when allowing for a QTL effect. The corresponding P value at this locus is given.

Discussion

The important findings in this study were that polymorphic microsatellite markers at the renin gene, the AT_1 receptor gene, the Liddle gene, and IGF-1 gene loci were linked to the phenotype systolic BP. The findings suggest that these sites are quantitative trait loci for BP in healthy humans. The linkage

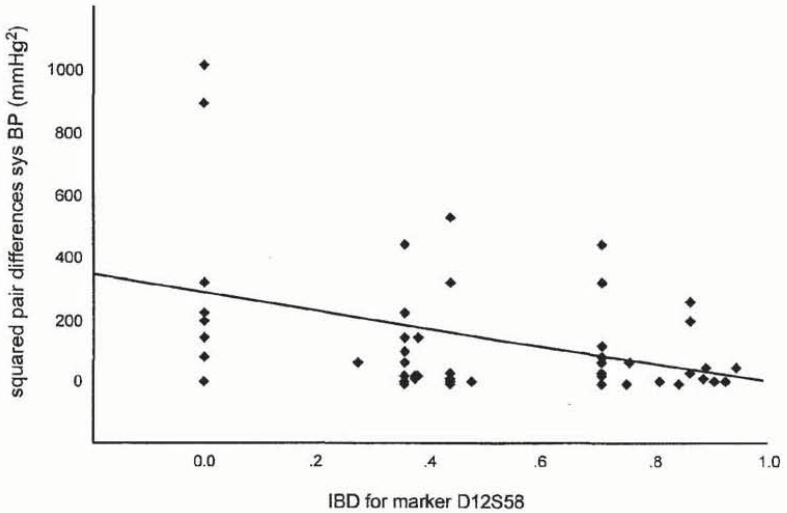


Figure 1. Ordinate shows the squared co-twin difference for systolic BP in dizygotic (DZ) twins. Abscissa displays the proportion of alleles identical by descent at the marker *D12S58*. Note the significant negative slope of the regression line ($P < 0.00001$), which is consistent with evidence for genetic linkage.

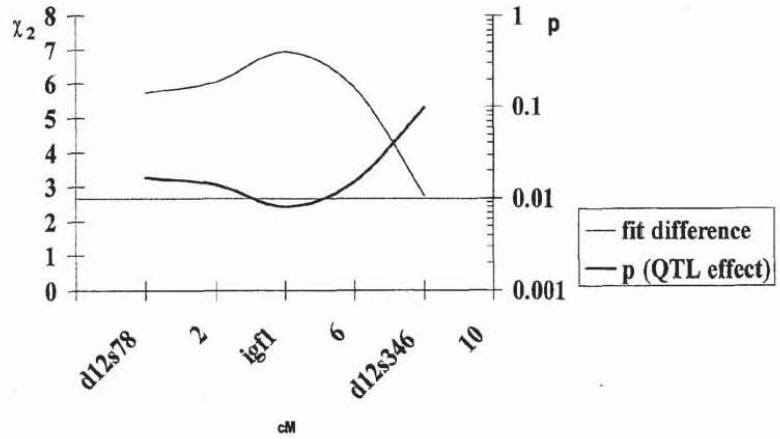


Figure 2. The quantitative trait loci (QTL) phenotype in the figure is cardiac mass according to the Penn formula. Left ordinate is the χ^2 and the top line is the fit difference between a model with and without the QTL effect. The bottom line shows the corresponding *P* value as given on the right ordinate.

with BP was strongest for the IGF-1 gene locus, where a QTL effect was demonstrable in all positions. We included the phenotype cardiac dimensions in our study and were able to show linkage between posterior wall thickness, septal thickness, and left ventricular mass index with the same markers at the IGF-1 gene. The linkage remained significant after correction for BP, suggesting that the findings may be independent of one another. These findings should not be interpreted as indicating "hypertension" or cardiac "hypertrophy" genes. BP and cardiac dimensions in our study are quantitative traits and not diseases.

Twin studies have been a classical tool in human genetics. Comparing the within- and among-pair differences for MZ and DZ twins for BP with ANOVA indicated the heritability of BP in earlier studies (3). Calculations can also be performed to estimate the degree of heritability as we performed here. Our heritability estimate was particularly high in part because of the narrow age range of our young subjects and perhaps because of the standardization of our BP measurements. Furthermore, DZ twins are a particularly powerful sib-pair model because of identical ages and a shared environment, at least in childhood. Interestingly, a quantitative trait locus for a closely defined reading disability has been described on chromosome 6, by means of sib-pair analysis including DZ twins (29). In that study, the power of DZ twins in the sib-pair analysis was aptly demonstrated; DZ twin sib pairs exhibited a LOD score twice that of affected siblings. This result would suggest that the sample size can be sharply reduced without a loss of power when DZ twin siblings are examined. The utility of DZ twins in the quantitative sib-pair linkage analysis approach to genes relevant to cardiovascular disease was recently demonstrated by Austin *et al.* (30), who found linkage between the microsomal triglyceride-transfer protein gene locus and plasma triglyceride concentrations, and also by Knoblauch *et al.* (31), who found linkage between the macrophage scavenger receptor gene locus and HDL cholesterol concentrations.

We found evidence for linkage between the renin gene locus and diastolic BP, which is more consistent than earlier findings (32). We also found no linkage with the ACE gene locus, which is also in agreement with earlier observations (33). However, we did find significant linkage to the AT_1 receptor gene locus on chromosome 3. At the angiotensinogen gene locus, we found very little suggestion of linkage to BP. Jeunemaitre *et al.* (6) and Caulfield *et al.* (7) described linkage between hypertension and the angiotensinogen gene in two large panels of hypertensive sibships. Conceivably, our sample size was too small to show an effect of this gene or, alternatively, the angiotensinogen gene locus plays no major role in normal BP regulation, a possibility we believe is unlikely. Wu *et al.* (19) were recently able to map a human systolic BP QTL near the lipoprotein lipase gene locus on chromosome 8p22. We were not able to find linkage at that locus in our subjects. Wu *et al.* (19) studied Taiwanese families with non-insulin-dependent diabetes mellitus. Differences in genetic backgrounds may account for our inability to find linkage at this site.

We were excited to find linkage between systolic BP and the Liddle syndrome locus on chromosome 16 (9,34). Our linkage markers, which are in proximity to both the β and γ subunit, suggest that additional linkage and association studies should be done in patients with hypertension to pursue this issue further. Recently, Melander and colleagues (35) described the βArg564X mutation as the cause for Liddle syndrome in a Swedish kindred. However, they were unable to identify polymorphisms in the gene, which were associated with hypertension or diabetic nephropathy. Persu *et al.* (36) found seven polymorphisms in this gene, which were more common in subjects of African origin, compared to Caucasian subjects.

Functionally significant properties could not be shown for these genetic variants. Baker *et al.* (37) recently studied 206 hypertensive black patients and 142 normotensive black control subjects in London, United Kingdom. Seventeen (8%) of the hypertensive blacks had the T594M mutation, compared with 2% of normotensive blacks. Those with the mutation had lower plasma renin activity, supporting the notion of increased sodium reabsorption. Thus, the T594M mutation may serve to explain some degree of salt sensitivity and hypertension in blacks. In addition, we found some evidence of linkage with diastolic BP in all three positions and the gene locus for autosomal-dominant hypertension and brachydactyly (10). The gene for this syndrome has not been cloned and the mechanism of the hypertension is unknown. This gene is located on the short arm of chromosome 12, as opposed to the IGF-1 gene, which is located on the long arm. These linkage findings are not interrelated.

We elected to examine the IGF-1 gene locus on chromosome 12 because of an earlier report by Frossard and Lestringant (38), who reported an association between a dimorphic site on chromosome 12 in the first intron of the human pancreatic phospholipase A2 gene and the clinical diagnosis of hypertension in three independent populations. They speculated from the results of their association studies that a QTL for BP could reside within 30 cM of this gene. Indeed, the most striking finding in our study was the identification of consistent linkage of systolic BP to the IGF-1 gene locus. Of course, we cannot be certain that the IGF-1 gene itself is exerting this effect, in contrast to some closely neighboring gene. However, the IGF-1 gene is a candidate. The role of IGF-1 in normal and pathologic cardiovascular physiology has recently been reviewed by Sowers (39). Andronico *et al.* (40) found that IGF-1 plasma levels were elevated in patients with essential hypertension, compared to normotensive control subjects and that a direct correlation between IGF-1 plasma levels and sodium/lithium countertransport existed in these subjects. They also observed that IGF-1 plasma concentrations and sodium/lithium countertransport were higher in patients with left ventricular hypertrophy, compared to those without this complication.

The same markers showing linkage between BP and the IGF-1 gene locus also gave a positive result for cardiac dimensions. Left ventricular hypertrophy is an independent risk factor for cardiac death, myocardial infarction, and congestive heart failure in patients with hypertension. Andronico *et al.* (40) found that BP load (systolic BP × heart rate) was correlated with IGF-1 plasma levels in patients with hypertension. IGF-1 mediates the functional activities of growth hormone, stimulates cell growth by autocrine or paracrine mechanisms, and induces cell proliferation in vascular smooth muscle. Enhanced production of IGF-1 has been found in the hearts of animals shortly after onset of experimental hypertension (41). However, the role of IGF-1 in BP regulation and the mediation of complications are by no means clear. Lembo *et al.* (42) generated mice homozygous for a site-specific insertional event that created a mutant IGF-1 allele. The mice had 30% of wild-type IGF-1 levels, but nevertheless had elevated BP and increased left ventricular contractility compared with wild-type mice. On the basis of increased adenylate cyclase activity the cardiomyocytes of these mice, the authors implicated cro talk between IGF-1 and β-adrenergic signaling pathways responsible.

Correcting the septal thickness, posterior wall thickness, a left ventricular mass index for possible effects of BP did 1 alter the relationship, raising the possibility that the obser tions are independent of one another. Reiss *et al.* (43) gen ated transgenic mice in which the cDNA for IGF-1β w placed under the control of the rat α-myosin heavy ch promoter. The mice have elevated IGF-1 serum concentratic and develop an increase in heart size. Furthermore, myocy from these mice demonstrate enhanced shortening velocity a cellular compliance (44). Growth hormone may increase l ventricular mass and improve ventricular performance in p tients with severe heart failure. A randomized controlled tr recently demonstrated that this therapy was effective, but or in patients who also exhibited an increase in IGF-1 ser concentrations (45). Our observations linking the IGF-1 ge locus to posterior wall thickness in healthy subjects is in acc with the conclusion that the gene for IGF-1 is an import candidate gene for susceptibility to cardiac hypertrophy.

We found consistent linkage with IGF gene locus only systolic, not diastolic, BP. Both measurements confer the sa degree of risk in terms of complications. Failure to find the QTL for diastolic BP is not necessarily inconsistent, but m instead merely underscore differences in systolic and diasto BP regulation. Interestingly, linkage with diastolic BP w found with the renin locus and the locus for autosomal-don nant hypertension and brachydactyly. Our findings, name that QTL for BP in healthy people exist at loci known to be importance to hypertension, may help in elucidating the mec anisms related to these genes or stimulating the search for n BP-relevant genes at these locations. The observation that t IGF-1 locus is also a QTL for cardiac dimensions suggests th particular attention should be directed to the IGF-1 gene. V present linkage data and no information on allelic varian Conceivably, either elevated or decreased IGF-1 levels unc different physiologic circumstances could be associated w elevated BP. Association studies, eventually coupled with pr tein expression studies, will be necessary to address this issu

We were also able to find linkage between the renin ge locus and BP. This finding should not be surprising, since opposed to angiotensin-converting enzyme, renin is recogniz as a rate-limiting step in angiotensin II production. Rapp *et* (46) found seven renin alleles in rats and identified alleles th cosegregated for BP. Jeunemaitre and colleagues (47) pe formed a sib-pair linkage analysis of renin gene haplotypes human hypertension and were not able to find support f linkage. The allelic concordance between the sib pairs w analyzed by identity by state relationships for 98 sib pai Their study may have not had sufficient power. Furthermo we treated BP as a quantitative trait. We performed no dire studies of hypertension. The phenotype "hypertension," wi which Pickering took so much issue, may be determined many genes each acting in concert (48). The QTL we ha found may have a bearing on hypertension; however, that fa

has not been shown for certain. Sequencing the corresponding genes with subsequent association analyses may reveal the allelic variations that contribute to hypertension.

Acknowledgments

These studies were supported by a Grant-in-Aid from the Bundesministerium für Bildung und Forschung (BMBF) (Klinisch-medizinischer Verbund). Dr. Busjahn is the recipient of a fellowship from the BMBF LPD 1995. Dr. Nagy was supported by a fellowship from the Deutscher Akademischer Austauschdienst.

References

1. Joint National Committee on Detection, Evaluation, and Treatment of High Blood Pressure (JNC-V): *Arch Intern Med* 153: 154–183, 1993
2. Weitz W: Zur Ätiologie der genuinen oder vaskulären Hypertension. *Z Klin Med* 96: 151–173, 1922
3. Ward R: Familial aggregation and genetic epidemiology of blood pressure. In: *Hypertension: Pathophysiology, Diagnosis, and Management*, edited by Laragh JH, Brenner BM, New York, Raven Press, 1990, pp 81–100
4. Lifton RP, Jeunemaitre X: Finding genes that cause human hypertension. *J Hypertens* 11: 231–236, 1993
5. Rapp JP, Wang SM, Dene H: A genetic polymorphism in the renin gene of Dahl rats cosegregate with blood pressure. *Science* 243: 542–544, 1989
6. Jeunemaitre X, Soubrier F, Kotelevsev YV, Lifton RP, Williams CS, Charru A, Hunt SC, Hopkins PN, Williams RR, Lalouel JM, Corvol P: Molecular basis of human essential hypertension: Role of angiotensinogen. *Cell* 71: 168–180, 1992
7. Caulfield M, Lavender P, Farrall M, Munroe P, Lawson M, Turner P, Clark AJ: Linkage of the angiotensinogen gene to the essential hypertension. *N Engl J Med* 330: 1629–1633, 1994
8. Tiret L, Bonnardeaux A, Poirier O, Ricard S, Marques-Vidal P, Evans A, Arveiler D, Luc G, Kee F, Ducimetiere P: Synergistic effects of angiotensin-converting enzyme and angiotensin-II type 1 receptor gene polymorphisms on risk of myocardial infarction. *Lancet* 344: 910–913, 1994
9. Shimkets RA, Warnock DG, Bositis CM, Nelson-Williams C, Hansson JH, Schambelan M, Gill JR, Ulick S, Milora RV, Findling JW, Canessa CM, Rossier BC, Lifton RP: Liddle's syndrome: Heritable human hypertension caused by mutations in the β subunit of the epithelial sodium channel. *Cell* 79: 407–414, 1994
10. Schuster H, Wienker TF, Bähring S, Bilginturan N, Toka HR, Neitzel H, Jeschke E, Tokan O, Gilbert D, Lowe A, Ott J, Haller H, Luft FC: Severe autosomal dominant hypertension and brachydactyly in a unique Turkish kindred maps to human chromosome 12. *Nat Genet* 13: 98–100, 1996
11. Reymer PW, Gagne E, Groenemeyer BE, Zhang H, Forsyth I, Jansen H, Seidell JC, Kromhout D, Lie KE, Kastelein J: A lipoprotein lipase mutation (Asn291Ser) is associated with reduced HDL cholesterol levels in premature atherosclerosis. *Nat Genet* 10: 28–33, 1995
12. Williams RR, Hunt SC, Hopkins PN, Wu LL, Hasstedt SJ, Berry TD, Barlow GK, Stults BM, Schumacher MC, Ludwig EH: Genetic basis of familial dyslipidemia and hypertension: 15-year results from Utah. *Am J Hypertens* 6: 319–327, 1993
13. Andronico G, Mangano MT, Nardi E, Mule G, Piazza G, Cerasola G: Insulin-like growth factor 1 and sodium-lithium countertransport in essential hypertension and in hypertensive left ventricular hypertrophy. *J Hypertens* 11: 1097–1101, 1993
14. Busjahn A, Faulhaber H-D, Viken RJ, Rose RJ, Luft FC: Genetic influences on blood pressure with the cold pressor test: A twin study. *J Hypertens* 14: 1195–1199, 1996
15. Busjahn A, Knoblauch J, Knoblauch M, Bohlender J, Menz M, Faulhaber H-D, Becker A, Schuster H, Luft FC: Angiotensin converting enzyme and angiotensinogen gene polymorphisms, plasma levels, and left ventricular size: A twin study. *Hypertension* 29: 165–170, 1997
16. Sahn DJ, DeMaria A, Kisslo J, Weymen A: Recommendations regarding quantitation in M-mode echocardiography: Results of a survey of echocardiographic measurements. *Circulation* 58: 1072–1083, 1978
17. Martin N, Boomsma D, Machin G: A twin-pronged attack on complex traits. *Nat Genet* 17: 387–392, 1997
18. Becker A, Busjahn A, Faulhaber H-D, Bähring S, Schuster H, Luft FC: Automated zygosity determination with microsatellites. *J Reprod Med* 42: 260–266, 1997
19. Wu D-A, Bu X, Warden CH, Shen DDC, Jeng C-Y, Sheu WHH, Fuh MMT, Katsuya T, Dzau VJ, Reaven GM, Lusis AJ, Rotter JI, Chen Y-DI: Quantitative trait locus mapping of human blood pressure to a genetic region at or near the lipoprotein lipase gene locus on chromosome 8p22. *J Clin Invest* 97: 2111–2118, 1996
20. Eaves LJ, Neale MC, Maes H: Multivariate multipoint linkage analysis of quantitative trait loci. *Behav Genet* 26: 519–525, 1996
21. Neale MC: *Mx: Statistical Modeling*, 4th Ed., Department of Psychiatry, Box 126 MCV, Richmond, VA 23298, 1997
22. Kruglyak L, Lander ES: Complete Multipoint Sib-Pair Analysis of Qualitative and Quantitative Traits. *Am J Hum Genet* 57: 439–454, 1995
23. Lander ES, Kruglyak L: Genetic dissection of complex traits: Guidelines for interpreting and reporting linkage results. *Nat Genet* 11: 241–246, 1995
24. S.A.G.E: Statistical Analysis for Genetic Epidemiology, Release 2.2. Computer program package available from the Department of Epidemiology and Biostatistics, Case Western Reserve University, Cleveland, OH, 1996
25. Blackwelder WC, Elston RC: Power and robustness of sib-pair linkage tests and extension to larger sibships. *Commun Statist-Theor Meth* 11: 449–484, 1982
26. Nance WE, Neale MC: Partitioned twin analysis: A power study. *Behav Genet* 19: 143–150, 1989
27. Fulker DW, Cherny SS: An improved multipoint sib-pair analysis of quantitative traits. *Behav Gen* 26: 527–532, 1996
28. Neale MC, Cardon LR: *Methodology for Genetic Studies of Twins and Families*, Dordrecht, The Netherlands, Kluwer Academic Publishers, 1992, p 496
29. Cardon LR, Smith SD, Fulker DW, Kimberling WJ, Pennington BF, DeFries JC: Quantitative trait locus for reading disability on chromosome 6. *Science* 266: 276–279, 1994
30. Austin MA, Talmud PJ, Luong L-A, Haddad L, Day INM, Newman B, Edwards LK, Krauss RM, Humphries SE: Candidate-gene studies of the atherogenic lipoprotein phenotype: A sib-pair linkage analysis of DZ women twins. *Am J Hum Genet* 62: 406–419, 1998
31. Knoblauch H, Busjahn A, Münter S, Nagy Z, Faulhaber H-D, Schuster H, Luft FC: Heritability analysis of lipids and three gene loci in twins link the macrophage scavenger receptor to high-density lipoprotein cholesterol concentrations. *Arterioscler Thromb Vasc Biol* 17: 2054–2060, 1997

32. Jeunemaitre X, Rigat B, Charru A, Houot AM, Soubrier F, Corvol P: Sib pair linkage analysis of renin gene haplotypes in human essential hypertension. *Hum Genet* 88: 301–306, 1992
33. Jeunemaitre X, Lifton RP, Hunt SC, Williams RR, Lalouel JM: Absence of linkage between the angiotensin-converting enzyme locus and human essential hypertension. *Nat Genet* 2: 72–75, 1992
34. Hansson JH, Nelson-Williams C, Suzuki H, Schild L, Shimkets R, Lu Y, Canessa C, Iwasaki T, Rossier B, Lifton RP: Hypertension caused by a truncated epithelial sodium channel g-subunit: Genetic heterogeneity of Liddle syndrome. *Nat Genet* 11: 76–82, 1995
35. Melander O, Orho M, Fagerudd J, Bengtsson K, Groop P-H, Mattiasson I, Groop L, Hulthen UL: Mutations and variants of the epithelial sodium channel gene in Liddle's syndrome and primary hypertension. *Hypertension* 31: 1118–1124, 1998
36. Persu A, Bargry P, Bassilana F, Houot A-M, Mengual R, Lazdunski M, Corvol P, Jeunemaitre X: Genetic analysis of the β subunit of the epithelial Na^+ channel in essential hypertension. *Hypertension* 32: 129–137, 1998
37. Baker EH, Dong YB, Sagnella GA, Rothwell M, Onipinia AK, Markandu ND, Cappuccio FP, Cook DG, Persu A, Corvol P, Jeunemaitre X, Carter ND, MacGregor GA: Association of hypertension with T594M mutation in β subunit of epithelial sodium channels in black people resident in London. *Lancet* 351: 1388–1392, 1998
38. Frossard PM, Lestringant GG: Association between a dimorphic site on chromosome 12 and clinical diagnosis of hypertension in three independent populations. *Clin Genet* 48: 284–287, 1995
39. Sowers JR: Insulin and insulin-like growth factor in normal and pathological cardiovascular physiology. *Hypertension* 29: 691–699, 1997
40. Andronico G, Mangano MT, Ferrara L, Lamanna D, Mule G, Cerasola G: Insulin-like growth factor 1 and pressure load in hypertensive patients. *Am J Hypertens* 9: 607–609, 1996
41. Wahlander H, Isgaard J, Jennische E, Friberg P: Left ventricular insulin-like growth factor 1 increases in early renal hypertension. *Hypertension* 19: 25–32, 1992
42. Lembo G, Rockman HA, Hunter JJ, Steinmetz H, Koch WJ, Ma L, Prinz-MP, Ross J Jr, Chien KR, Powell-Braxton L: Elevated blood pressure and enhanced myocardial contractility in mice with severe IGF-1 deficiency. *J Clin Invest* 98: 2648–2655, 1996
43. Reiss K, Cheng W, Ferber A, Kajstura J, Li P, Li B, Olivetti G, Homcy CJ, Baserga R, Anversa P: Overexpression of insulin-like growth factor-1 in the heart is coupled with myocyte proliferation in transgenic mice. *Proc Natl Acad Sci USA* 93: 8630–8635, 1996
44. Redaelli G, Malhotra A, Li B, Li P, Sonnenblick EH, Hofmann PA, Anversa P: Effects of constitutive overexpression of insulin-like growth factor-1 on the mechanical characteristics and molecular properties of ventricular myocytes. *Circ Res* 82: 594–603, 1998
45. Osterziel KJ, Strohm O, Schuler J, Friedrich M, Hänlein D, Willenbrock R, Anker SD, Poole-Wilson PA, Ranke MB, Dietz R: Randomised, double-blind, placebo-controlled trial of human recombinant growth hormone in patients with chronic heart failure due to dilated cardiomyopathy. *Lancet* 351: 1233–1237, 1998
46. Rapp JP, Dene H, Deng AV: Seven renin alleles in rats and their effects on blood pressure. *J Hypertens* 12: 349–355, 1994
47. Jeunemaitre X, Rigat B, Charru A, Houot AM, Soubrier F, Corvol P: Sib pair linkage analysis of renin gene haplotypes in human essential hypertension. *Hum Genet* 88: 301–306, 1992
48. Luft FC: Molecular genetics of human hypertension. *J Hypertens* 16: 1871–1878, 1998

β-2 Adrenergic Receptor Gene Variations, Blood Pressure, and Heart Size in Normal Twins

Andreas Busjahn, Guo-Hua Li, Hans-Dieter Faulhaber, Magda Rosenthal, Albert Becker, Eva Jeschke, Herbert Schuster, Bernd Timmermann, Margret R. Hoehe, Friedrich C. Luft

Abstract—Genetic variability, which influences cardiovascular phenotypes in normal persons, is likely to be relevant to cardiovascular disease. We studied normal monozygotic and dizygotic twins and found strong genetic influences on blood pressure and heart size. We then relied on the dizygotic twins and their parents to apply molecular genetic techniques. We performed a linkage analysis with markers close to the β-2 adrenergic receptor (AR) gene locus in the dizygotic twins and their parents and found strong evidence for linkage to the quantitative traits of blood pressure and heart size. We then used allele-specific polymerase chain reaction to genotype the subjects further. We performed an association analysis and found that 4 functionally relevant polymorphisms in the β-2 AR gene, namely Arg16/Gly, Gln27/Glu, Thr164/Ile, and a variant in the promoter region (−47C/T), were variably associated with blood pressure and heart size differences but were in linkage dysequilibrium with each other. A subsequent conditional analysis suggested that the Arg16/Gly polymorphism exerted the predominant effect. These findings underscore the importance of the β-2 AR gene to blood pressure regulation, heart size, and probably to the development of hypertension. We suggest that a combined linkage and association approach will elucidate the genetic variability influencing blood pressure and other cardiovascular phenotypes. **(*Hypertension*. 2000;35:555-560.)**

Key Words: receptors, adrenergic, beta ■ genetics ■ hypertension, genetic ■ twins ■ blood pressure

The β-2 adrenergic receptor (β-2 AR) has been implicated in the pathogenesis of hypertension, both on the basis of studies suggesting altered β-2–mediated vasodilation[1] and on the basis of molecular genetic association studies.[2,3] Recently, Kotanko et al[4] found an association between the Arg16/Gly polymorphism in the β-2 AR gene and hypertension in an African Caribbean population. They showed that the Gly16 allele was more common in hypertensive subjects than in normotensive African Caribbean control subjects. Since the Gly16 allele indicates an increased propensity for downregulation of the receptor,[5] the authors raised the possibility that an impaired vasodilation in peripheral arteries in response to β-2 AR agonists may play a role in the hypertension of individuals carrying the Gly16 allele. We subsequently examined the firstborn normotensive adult children of couples documented to be normotensive or hypertensive in the Bergen Blood Pressure Study.[6] Offspring of 2 hypertensive parents had higher blood pressures and a preponderance of the Arg16 allele compared with offspring of 2 normotensive parents. To further examine the genetic variability at the β-2 AR locus and its relevance for the cardiovascular system in northern Europeans, we performed a combined linkage and association study in normotensive twin subjects. We used allele-specific polymerase chain reaction (PCR), which allowed us to examine other polymorphisms in the β-2 AR gene.

Methods

We recruited 166 pairs of twins (monozygotic [MZ] 100 and dizygotic [DZ] 66) by advertisement to participate in studies involving blood pressure regulation and cardiovascular phenotypes.[7,8] We recruited the parents of the DZ twins and genotyped them as well, to permit an identity by descent (IBD) analysis. The subjects were all Germans, white subjects recruited from various parts of Germany. The protocol was approved by the university committee on the protection of human subjects, and written informed consent was obtained from all participants. Blood was obtained for the determination of zygosity and other molecular genetic studies from all the twins and the parents of the DZ twins. Each participant underwent a medical history and physical examination. Subjects taking antihypertensive medication or being affected by any other chronic medical illness were excluded. Blood pressure was measured after 5 minutes of rest in the sitting position with a standardized mercury sphygmomanometer (World Health Organization criteria) by an experienced physician. Two measurements were obtained 2 minutes apart. The same procedure was performed after the subjects were supine for 5 minutes and after 5 minutes of upright posture. The mean values of the 2 determinations were recorded. Norepinephrine was measured with high-performance liquid chromatography and electrochemical detection. M-mode and 2-dimensional echocardiograms were recorded with patients in the left lateral decubitus position. Interventricular septal thickness and posterior wall thickness were measured in all patients as described earlier.[8]

Received August 27, 1999; first decision September 27, 1999; revision accepted October 8, 1999.

From Franz Volhard Clinic and Max Delbrück Center for Molecular Medicine, Medical Faculty of the Charité, Humboldt University of Berlin, Germany.

Correspondence to Friedrich C. Luft, Franz Volhard Clinic, Wiltberg Strasse 50, 13122 Berlin, Germany. E-mail luft@fvk-berlin.de

Hypertension **is available at http://www.hypertensionaha.org**

TABLE 1. Demographic Data, Phenotypic Values (Mean±SD), Correlations (*r*), Heredity Estimates (a^2), and Results of Linkage Analysis with the β-2 Locus

Phenotype	MZ Twins	DZ Twins	a^2 (r_{MZ}/r_{DZ})	*P* (Linkage)
n	200	132		
Age, y	29±12	31±12		
Sex, M/F	52/148	85/47		
Height, cm	169±8	170±8		
Weight, kg	65±11	67±12		
BMI, kg/m	22.4±3.5	22.8±3.4		
Systolic BP recumbent, mm Hg	128±17	124±14	0.69 (0.69/0.31)	0.001
Diastolic BP recumbent, mm Hg	71/12	71/11	0.66 (0.66/0.42)	0.001
Systolic BP sitting, mm Hg	125±16	123±13	0.74 (0.74/0.38)	NS
Diastolic BP sitting, mm Hg	73±11	73±10	0.72 (0.72/0.51)	0.001
Systolic BP standing, mm Hg	124±15	122±14	0.67 (0.66/0.48)	0.001
Diastolic BP standing, mm Hg	80±10	79±10	0.64 (0.63/0.40)	NS
Posterior wall thickness, mm	8.7±1.6	8.6±1.6	0.48 (0.48/0.26)	NS
Septum, mm	8.9±1.7	8.8±1.6	0.64 (0.64/0.37)	0.001
Left ventricular mass, mm^3	165±50	176±60	0.68 (0.68/0.27)	0.001
Norepinephrine, pmol/L*	54.7/21.2	58.1/23.1	0.30 (0.60/0.45)	0.001

BMI indicates body mass index; BP, blood pressure.
*pg/mL, 5.458 pmol/L.

For linkage analysis, 3 microsatellite markers spanning 2.3 cM around the β-2 locus on chromosome 5 (D5S413, D5S2090, and D5S2013) were analyzed with the use of the ABI genotyping system. Our techniques for zygosity testing and genotyping of microsatellites have been previously described.[9,10] We genotyped the functionally relevant polymorphisms of the β-2 AR by means of allele-specific polymerase chain reaction (PCR), as described elsewhere.[6]

Statistical analysis was conducted with the use of the SPSS program. Association analysis was based on quantitative measures. Blood pressure values and all other phenotypes were adjusted for sex and age by multiple linear regression. Cardiac dimensions were also adjusted for blood pressure–related influences. Two different approaches were applied. Phenotypic values were compared between groups defined by their genotype with the use of ANOVA for all 4 polymorphisms independently. To increase the power for the association analysis, mean scores of pairs of MZ twins were included together with 1 randomly selected member of the DZ pairs.[11] Because this approach might be prone to false-positive results as the result of population stratification, a second analysis based on sib-pair data was carried out.[12] To test for association, structural equation modeling was used to obtain maximum likelihood estimates for the allelic effect, based on within-family and between-family differences. Significance was tested by computing nested models and comparing the log-likelihood between models. To test for stratification effects, both estimates of allelic effect are constrained to be equal. In the absence of stratification, both estimates were then set to zero. In the presence of stratification effects, setting only the within-family estimate to zero provides an unbiased test for true allelic association.

Haplotypes could not be constructed for all subjects. Furthermore, for polymorphisms in close linkage disequilibrium, not all informative combinations were present in the sample. To estimate the influence of single polymorphisms independent from each other, conditional analyses were carried out within subgroups differing for 1 polymorphism while being equally homozygous for a second polymorphism. This approach controlled for possible influences of the second polymorphism. For the association analysis we accepted a value of $P<0.05$ as significant.

For linkage analysis, twin pairs were selected and used as ordinary sib-pairs but with the advantage of perfect age matching and reduced environmental variation affecting the phenotype. We assessed linkage for blood pressure as a continuous trait. Analysis was done by using a structural equation modeling approach,[13] as implemented in the MX-package developed by Neale.[14] If the locus under study is a quantitative trait locus (QTL), phenotypic similarity of sibs (measured by their covariance) should increase with the number of alleles shared IBD. For each sib-pair, the proportion of alleles IBD for the given locus is calculated on the basis of parental genotypes, with the use of a multipoint approach, as implemented in MAPMAKER/SIBS.[15] To test for a QTL effect, the difference in model fit for models with and those without a QTL effect is calculated as a χ^2 statistic. Testing of multiple correlated phenotypes was used to verify results; thus no adjustment for multiple testing was performed. Because we used a candidate gene approach, we accepted a value of $P<0.01$ to test for significant linkage in accordance with the criteria defined by Lander and Kruglyak.[16] Parameters of the quantitative genetic models were estimated on the basis of variance/covariance matrixes by structural equation modeling[17] with the use of the MX program.[18] The variability of any given phenotype within a population can be decomposed into genetic influences, environmental influences shared by the twins within a family, and effects of random environment.

Results

Demographic data, blood pressure values, heredity estimates of blood pressure, and echocardiographic variables in 200

TABLE 2. Position, Consequence, and Genotype Frequencies for Polymorphisms

Position (Nucleotide base)	Polymorphism/ Frequency	Amino Acid Change/Position
−47	C/T (38%/62%)	(regulatory region)
+46	A/G (47%/53%)	Arg/Gly/16
+79	C/G (61%/49%)	Gln/Glu/27
+491	G/A (99%/1%)	Thr/Ile/164

TABLE 3. Frequencies for Genotype Combinations

Genotype	Arg16/Gly Arg/Arg	Arg/Gly	Gly/Gly	P
−47CT				
C/C	0 (0%)	3 (0.9%)	38 (17.8%)	
C/T	3 (1.4%)	56 (26.3%)	24 (11.3%)	
T/T	55 (25.8%)	26 (12.2%)	9 (4.2%)	0.01
	Glu27/Gln Glu/Glu	Glu/Gln	Gln/Gln	
−47CT				
C/C	0 (0%)	1 (0.5%)	39 (18.3%)	
C/T	1 (0.5%)	78 (36.6%)	4 (1.9%)	
T/T	87 (40.8%)	3 (1.4%)	0 (0%)	0.01
	Thr164/Ile Thr/Thr	Thr/Ile	Ile/Ile	
−47CT				
C/C	40 (18.8%)	0 (0%)	0 (0%)	
C/T	79 (37.1%)	3 (1.4%)	1 (0.5%)	
T/T	88 (41.3%)	2 (0.9%)	0 (0%)	NS
	Glu27/Gln Glu/Glu	Glu/Gln	Gln/Gln	
Arg16/Gly				
Arg/Arg	55 (25.8%)	3 (1.4%)	0 (0%)	
Arg/Gly	25 (11.7%)	58 (27.2%)	1 (0.5%)	
Gly/Gly	8 (3.8%)	21 (9.9%)	42 (19.7%)	0.01
	Thr164/Ile Thr/Thr	Thr/Ile	Ile/Ile	
Arg16/Gly				
Arg/Arg	58 (27.2%)	0 (0%)	0 (0%)	
Arg/Gly	83 (39.0%)	1 (0.5%)	0 (0%)	
Gly/Gly	66 (31.0%)	4 (1.9%)	1 (0.5%)	NS
	Thr164/Ile Thr/Thr	Thr/Ile	Ile/Ile	
Glu27/Gln				
Glu/Glu	86 (40.4%)	2 (0.9%)	0 (0%)	
Glu/Gln	79 (37.1%)	2 (0.9%)	1 (0.5%)	
Gln/Gln	42 (19.7%)	1 (0.5%)	0 (0%)	NS

MZ and 132 DZ twins are given in Table 1. There were no significant differences between MZ and DZ twins for any of the variables examined. Systolic and diastolic blood pressures were heritable. The echocardiographic parameters also demonstrated strong evidence of heritability. The multipoint linkage analysis gave significant ($P<0.001$) results for systolic blood pressure recumbent and standing, diastolic blood pressure sitting and recumbent, and left ventricular mass index, as shown in Table 1. These data establish the β-2 AR gene locus as a QTL for blood pressure and heart size in normal humans.

We next performed allele-specific PCR for 4 polymorphisms resulting in an amino acid exchange. The gene frequencies are given in Table 2. The test for Hardy-Weinberg equilibrium gave evidence for an excess of homozygous individuals for polymorphisms in the 5′ leader cistron and the first extracellular loop of the gene (−47C/T, Arg16/Gly, Gln27/Glu), whereas the polymorphism in the fourth transmembrane domain (Thr164/Ile) showed no deviation from expected frequencies. We performed an analysis to assess the interdependency of the polymorphisms, as shown in Table 3. The strongest linkage was found between polymorphisms in close spatial relation to one another, namely −47C/T, Arg16/Gly, and Gln27/Glu. Thr164/Ile was independent of −47C/T, Gln27/Glu, and Arg16/Gly.

Tables 4 and 5 show the results of the association analysis. Probability values for both ANOVA and maximum likelihood estimation are given, although the results with the 2 approaches were relatively similar. Arg16/Gly and Gln27/Glu showed strong associations to systolic blood pressure and norepinephrine levels as well as with septum and posterior wall thickness. Thr164/Ile was associated with recumbent systolic blood pressure (ANOVA) and all cardiac dimensions. The associations with cardiac dimensions for the polymorphisms remained significant, even when corrected for systolic blood pressure by multiple linear regression analysis (data not shown). Homozygous Ile164/Ile was observed in only 1 person. The biallelic polymorphism in the regulatory region was weakly associated with systolic blood pressure, norepinephrine levels, and septum thickness.

Table 6 shows the results of a conditional analysis for the Arg16/Gly and Gln27/Glu polymorphisms. In subjects homozygous for Gly/Gly, the Gln27/Glu polymorphism showed no effect on any of the phenotypes. In contrast, when we examined subjects with the Gln27/Glu polymorphism who were homozygous Gln/Gln, Arg16/Arg subjects differed from Gly16/Gly subjects for all 3 blood pressure values and for cardiac dimensions. These results favor the interpretation that the Arg16/Gly polymorphism is more likely to be responsible for the effects on the phenotypes.

Discussion

The important findings in this study were that the β-2 AR gene locus is linked to the quantitative traits systolic blood pressure, diastolic blood pressure, and cardiac size, indicating that this gene locus is a QTL for blood pressure and heart size in normotensive individuals. Furthermore, the 4 polymorphisms in the β-2 AR gene that we examined, namely Arg16/Gly, Gln27/Glu, Thr164/Ile, and a variant in the promoter region (−47C/T), were all variably associated with systolic blood pressure and heart size differences in these normal subjects, whereas 3 of the 4 were associated with differences in norepinephrine levels. We report on 4 biallelic polymorphisms in the β-2 AR gene, which result in amino acid substitutions.[18] A fifth, Val34/Met, was not encountered in our subjects. We believe that our combined linkage-association approach is unique and strongly supports an important role for the β-2 AR gene in blood pressure regulation. The significant associations we observed were for systolic, not diastolic, blood pressure. This finding should not

TABLE 4. Three Biallelic Polymorphisms in the β-2 AR Gene Resulting in Amino Acid Change

Phenotype	Arg16/Arg/Arg	Gly Arg/Gly	Gly/Gly	*P*, ANOVA	*P*, ML
n, subjects	58	84	71		
Systolic BP recumbent	131±15	125±16	123±14	<0.05	<0.05
Systolic BP sitting	128±14	124±14	122±14	<0.05	<0.05
Systolic BP standing	127±14	123±14	120±14	<0.05	<0.05
Norepinephrine	58.4±23.8	61.9±23.5	50.7±17.0	<0.05	<0.05
Septum thickness	9.3±1.6	8.9±1.6	8.6±1.4	<0.05	<0.05
Posterior wall thickness	8.9±1.4	8.8±1.5	8.4±1.3	0.08	<0.05
Left ventricular mass	174±32	173±45	167±42	NS	<0.05
	Gln27/Glu/Glu	Glu Glu/Gln	Gln/Gln	*P*, ANOVA	*P*, ML
n, subjects	88	82	43		
Systolic BP recumbent	130±14	124±16	123±17	<0.05	<0.05
Systolic BP sitting	127±13	122±15	122±16	0.053	NS
Systolic BP standing	126±13	121±15	120±14	<0.05	<0.05
Norepinephrine	61.2±24.0	56.2±21.6	50.4±16.1	<0.05	<0.05
Septum thickness	9.3±1.6	8.6±1.5	8.7±1.4	<0.05	<0.05
Posterior wall thickness	8.9±1.5	8.6±1.4	8.4±1.3	NS	<0.05
Left ventricular mass	176±40	166±42	172±38	NS	NS
	Thr164/Thr/Thr	Ile Thr/Ile	Ile/Ile*	*P*, *t* test	*P*, ML
n, subjects	208	5	(1)		
Systolic BP recumbent	126±16	122±4		<0.05	NS
Systolic BP sitting	124±14	124±5		NS	NS
Systolic BP standing	123±14	124±8		NS	NS
Norepinephrine	57.2±22.2	58.3±18.3		NS	NS
Septum thickness	8.9±1.6	10.3±1.9		<0.05	<0.05
Posterior wall thickness	8.6±1.4	9.7±2.0		<0.05	<0.05
Left ventricular mass	170±39	220±60		<0.05	<0.05

BP, blood pressure.
The association analysis was performed both with ANOVA and a maximum likelihood (ML) approach.
*This subject was deleted from the analysis.

be surprising, because earlier studies indicate that systolic and diastolic blood pressures may be influenced by different genes.[7] Furthermore, systolic blood pressure may be more a function of aortic elasticity and pulse-wave velocity, whereas diastolic blood pressure may be more a function of peripheral vascular resistance.[19]

The significant deviation from Hardy-Weinberg equilibrium detected in our sample prompted us to test allele frequencies from other studies for the Arg16/Gly polymorphisms. In the Bergen Blood Pressure study[6] we found the same excess of homozygous individuals as in this twin study, but because of sample size this deviation did not reach statistical significance. Another sample of healthy subjects[5] showed a deviation from expected genotype frequencies pointing in the same direction. Interestingly, the hypertensive persons described by Kotanko et al[4] also deviated significantly from Hardy-Weinberg equilibrium. The confirmation of excess homozygosity in independent samples makes us confident that this finding is neither a genotyping error nor an effect specific for twin subjects. At this point, we can only speculate that there may be some disadvantage in having 2 different variants of the β-2 AR as the result of heterozygosity.

The Gly16 variant has been shown to represent a β-2 AR, which is more likely to be downregulated in response to β-2 agonists.[5] The Gly16 variant was more common in hypertensive compared with normotensive African Caribbeans than the Arg16 variant and was also found to indicate a lesser propensity to vasodilatation in response to salbutamol infusion in normotensive white subjects from Austria.[20] Lang et al[21] observed that forearm blood flow responses to isoproterenol were markedly attenuated in normotensive black subjects compared with white subjects, indicating a blunting of vasodilation mediated by the β-2 AR. In that study, the β-2 AR alleles were not examined. Possibly, the Gly16 variant is associated with salt sensitivity; however, that hypothesis has not been prospectively tested.

Arg16 wild-type subjects had recumbent, sitting, and standing systolic blood pressures 7 to 8 mm Hg higher than homozygous Gly16 subjects, whereas heterozygous subjects were intermediate. Gly16 was also associated with a lesser cardiac septum thickness and lower norepinephrine levels.

TABLE 5. Biallelic Polymorphism in the Regulatory Region of the β-2 AR Gene

	−47C/T				
Phenotype	WW	WP	PP	*P*, ANOVA	*P*, ML
n, subjects	40	83	90		
Systolic BP recumbent	124±17	124±16	129±14	<0.05	NS
Systolic BP sitting	123±16	122±15	126±13	0.08	NS
Systolic BP standing	120±14	122±15	126±13	0.06	NS
Norepinephrine	50.9±17.0	55.9±21.4	61.2±23.8	<0.05	NS
Septum thickness	8.7±1.4	8.7±1.5	9.2±1.6	<0.05	<0.05
Posterior wall thickness	8.3±1.3	8.6±1.4	8.9±1.5	NS	<0.05
Left ventricular mass	169±37	168±43	175±39	NS	NS

BP, blood pressure; ML, maximum likelihood.

We have no explanation for the discrepancy between our observations and those reported earlier for African Caribbeans. Ethnic differences may be responsible and suggest that the hypertensive mechanisms may be quite different in black Africans and white subjects. Our conditional analysis gave support to the notion that the Arg16/Gly polymorphism is responsible for the effects on blood pressure and heart size rather than the Gln27/Glu polymorphism. Thus differences in Gln27/Glu distributions between the 2 populations would not serve to explain the discrepancy. Nevertheless, genotyping the African Caribbean population for other β-2 AR polymorphisms would be of interest.

The Glu27/Gln variant was the second most common polymorphism we encountered. This variant has been associated with an increase in IgE concentrations in the serum of asthmatic families.[22] In contrast to Arg16/Gly, the Glu27/Gln variant is apparently resistant to downregulation, whereas the combination mutant downregulates to the same extent as the Gly16 variant. The most functionally altered β-2 AR is found in persons with a Thr-to-Ile switch at amino acid 164. This receptor exhibits a small decrease in binding affinity for agonists but a substantial decrease in basal and epinephrine-stimulated adenylyl cyclase activities caused by defective coupling of the receptor to the stimulatory G protein G_s and impaired agonist-promoted sequestration.[23,24] In transgenic mice expressing either the wild-type Thr164 or the Ile164 mutation in the heart, the Ile164 mice displayed depressed contractile function compared with controls.[25] Recently, Liggett et al[26] were able to show that patients with heart failure who were heterozygous for the Ile164 mutation had a strikingly worse survival compared with patients with the wild-type Thr at this position. Liggett et al[26] had no homozygous Ile164 subjects in their study. We encountered 1 such subject, who was clinically well. Ile164 may only become a relevant risk factor when persons develop congestive heart failure but may not necessarily be associated with conditions engendering heart failure.

We first observed the −47C/T polymorphism of the 5′ leader cistron in the β-2 AR gene, which results in either Arg or Cys being encoded at the terminal amino acid of the receptor peptide, in our subjects from the Bergen Blood Pressure Study.[27] McGraw et al[28] recently reported that the Cys variant results in increased β-2 AR expression. Thus the −47C/T polymorphism may represent the genetic basis of variable physiological sympathetic responses or variations in cardiovascular phenotypes.

We cannot state for certain which polymorphism we examined is responsible for the association with blood pressure, cardiac dimensions, and norepinephrine concentrations, nor can we speculate whether or not the functional polymorphisms exert independent effects. Additional interactions between these polymorphisms may be elucidated by larger

TABLE 6. Conditional Analysis for Arg16/Gly and Gln27/Glu

	Within Gly16/Gly			Within Gln27/Gln		
Phenotype	Glu27/Glu	Gln27/Gln	P	Arg16/Arg	Gly16/Gly	*P*
n (subjects)	8	42		55	8	
Systolic BP recumbent	126±7	124±18	NS	132±17	126±7	<0.05
Systolic BP sitting	120±7	122±17	NS	128±15	120±7	<0.05
Systolic BP standing	122±8	120±16	NS	128±15	122±8	<0.05
Norepinephrine	58.6±15.6	48.6±15.9	NS	57.2±32.1	58.6±15.6	NS
Septum thickness	8.8±1.9	8.7±1.4	NS	9.3±1.7	8.8±1.9	<0.05
Posterior wall thickness	8.6±1.9	8.4±1.3	NS	8.9±1.4	8.6±1.9	<0.05
Left ventricular mass	173±65	162±48	NS	177±53	173±65	<0.05

BP, blood pressure.

The analysis compares persons homozygous for Gly16/Gly in terms of Gln27/Gln as well as persons homozygous for Gln27/Gln in terms of Arg16/Gly.

studies with complex haplotype analyses. Nevertheless, our study shows that at least 2 functionally relevant, β-2 AR polymorphisms are associated with blood pressure, cardiac dimensions, and norepinephrine levels in normal healthy dizygotic twin subjects. Furthermore, the β-2 AR gene locus is a QTL for these variables in normal humans. Finally, our study suggests that the Gly16 rather than the Arg16 variant is associated with lower blood pressure and a lesser risk to develop hypertension in white subjects. Longitudinal outcome studies incorporating haplotype analyses will be elucidative in this regard.

Acknowledgments

Contributions of Andreas Busjahn and Guo-Hua Li were equal. This study was supported by a grant-in-aid from the Bundesministerium für Bildung und Forschung, Bonn, FRG.

References

1. Skrabal F, Kotanko P, Luft FC. Minireview: inverse regulation of α-2 and β-2 adrenoceptors in salt-sensitive hypertension: an hypothesis. *Life Sci*. 1989;45:2061 2076.
2. Svetkey L-P, Timmons P-Z, Emovon O, Anderson N-B, Preis L, Chen Y-T. Association of hypertension with the β-2 and α 2c10 adrenergic receptor genotype. *Hypertension*. 1996;27:1210 1215.
3. Svetkey L-P, Chen Y-T, McKeown S-P, Preis L, Wilson A-F T-I. Preliminary evidence of linkage of salt sensitivity in black Americans at the β 2-adrenergic receptor locus. *Hypertension*. 1997;29:918 922.
4. Kotanko P, Binder A, Tasker J, DeFreitas P, Kamdar S, Clark A-J-L, Skrabal F, Caulfield M. Essential hypertension in African Caribbeans associates with a variant of the β-2 adrenoceptor. *Hypertension*. 1997; 30:773 776.
5. Turki J, Pak J, Green SA, Martin RJ, Liggett SB. Genetic polymorphisms of the β2-adrenergic receptor in nocturnal and nonnocturnal asthma: evidence that Gly16 correlates with the nocturnal phenotype. *J Clin Invest*. 1995;95:1635 1641.
6. Timmermann B, Rune M, Luft FC, Gerdts E, Busjahn A, Omvik P, Guo-Hua L, Schuster H, Wienker T-F, Hoehe M, Lund-Johansen P. β-2 adrenoceptor genetic variation is associated with genetic predisposition to essential hypertension: the Bergen Blood Pressure Study. *Kidney Int*. 1998;53:1455 1460.
7. Busjahn A, Faulhaber H-D, Viken RJ, Rose RJ, Luft FC. Genetic influences on blood pressure with the cold pressor test: a twin study. *J Hypertens*. 1996;14:1195 1199.
8. Busjahn A, Knoblauch J, Knoblauch M, Bohlender J, Menz M, Faulhaber H-D, Becker A, Schuster H, Luft FC. Angiotensin converting enzyme and angiotensinogen gene polymorphisms, plasma levels, and left ventricular size: a twin study. *Hypertension*. 1997;29:165 170.
9. Becker A, Busjahn A, Faulhaber H-D, Bähring S, Schuster H, Luft FC. Automated twin zygosity determination with microsatellites. *J Reprod Med*. 1997;42:260 266.
10. Knoblauch H, Busjahn A, Münter S, Nagy Z, Faulhaber H-D, Schuster H, Luft FC. Heritability analysis of lipids and 3 gene loci in twins link the macrophage scavenger receptor to high-density lipoprotein cholesterol concentrations. *Arterioscler Thromb Vasc Biol*. 1997;17:2054 2060.
11. Miller MB. Monozygotic twins increase the power of genetic association studies of complex phenotypes. *Behav Gen*. 1998;28:476. Abstract.
12. Fulker DW, Cherny SS, Sham PC, Hewitt JK. Combined linkage and association sib-pair analysis for quantitative traits. *Am J Hum Genet*. 1999;64:259 267.
13. Busjahn A, Knoblauch H, Faulhaber H-D, Uhlmann R, Hoehe M, Schuster H, Luft FC. The QT interval is linked to 2 long-QT syndrome loci in normal subjects. *Circulation*. 1999;99:3161 3164.
14. Neale MC. *Mx: Statistical Modeling*. Richmond, Va: Department of Psychiatry. 4th ed 1997.
15. Kruglyak L, Lander ES. Complete multipoint sib-pair analysis of qualitative and quantitative traits. *Am J Hum Genet*. 1995;57:439 454.
16. Lander ES, Kruglyak L. Genetic dissection of complex traits: guidelines for interpreting and reporting linkage results. *Nat Genet*. 1995;11: 241 246.
17. Neale MC, Cardon LR. *Methodology for Genetic Studies of Twins and Families*. Dordrecht, Netherlands: Kluwer Academic Publishers; 1992.
18. Liggett S. Functional properties of human β2-adrenergic receptor polymorphisms. *NIPS*. 1995;10:265 273.
19. Safar ME, London GM, Asmar R, Frohlich ED. Recent advances on large arteries in hypertension. *Hypertension*. 1998;32:156 161.
20. Gratze G, Fortin J, Labugger R, Binder A, Kotanko P, Timmermann B, Luft FC, Hoehe M, Skrabal F. β-2 Adrenergic receptor variants affect agonist-induced vasodilatation in normotensive Caucasians. *Hypertension*. 1999;33:1425 1430.
21. Lang C-C, Stein C-M, Brown R-M, Deegan R, Nelson R, He H-B, Wood M, Wood A-J. Attenuation of isoproterenol-mediated vasodilation in blacks. *N Engl J Med*. 1995;333:155 160.
22. Dewar JC, Wilkinson J, Wheatley A, Thomas NS, Doull I, Morton N, Harvey JF, Liggett SB, Holgate ST, Hall IP. The glutamine 27 β2-adrenoceptor polymorphism is associated with elevated IgE levels in asthmatic families. *J Allergy Clin Immunol*. 1997;100:261 265.
23. Green SA, Cole G, Jacinto M, Innis M, Liggett SB. A polymorphism of the human β2-adrenergic receptor within the fourth transmembrane domain alters ligand binding and functional properties of the receptor. *J Biol Chem*. 1993;268:23116 23121.
24. Green S, Turki J, Innis M, Liggett SB. Amino-terminal polymorphisms of the human b2-adrenergic receptor impart distinct agonist-promoted regulatory properties. *Biochemistry*. 1994;33:9414 9419.
25. Turki J, Lorenz JN, Green SA, Donnelly ET, Jacinto M, Liggett SB. Myocardial signalling defects and impaired cardiac function of a human β2-adrenergic receptor polymorphism expressed in transgenic mice. *Proc Natl Acad Sci U S A*. 1996;89:10483 10488.
26. Liggett SB, Wagoner LE, Craft LL, Hornung RW, Hoit BD, McIntosh TC, Walsh RA. The Ile164 β2-adrenergic receptor polymorphism adversely affects the outcome of congestive heart failure. *J Clin Invest*. 1998;102:1534 1539.
27. Timmermann B, Li G-H, Luft FC, Lund-Johansen P, Skrabal F, Hoehe M. Novel DNA sequence differences in the beta2-adrenergic receptor gene promoter region. *Hum Mutat*. 1998;11:343 344.
28. McGraw DW, Forbes SL, Kramer LA, Liggett SB. Polymorphisms of the 5[prime] leader cistron of the human β2-adrenergic receptor regulate receptor expression. *J Clin Invest*. 1998;102:1927 1932.

Am. J. Hum. Genet. 66:157–166, 2000

A Cholesterol-Lowering Gene Maps to Chromosome 13q

Hans Knoblauch,[1,*] Bertram Müller-Myhsok,[2,*] Andreas Busjahn,[1] Liat Ben Avi,[3] Sylvia Bähring,[1] Heike Baron,[1] Simon C. Heath,[5] Regina Uhlmann,[1] Hans-Dieter Faulhaber,[1] Shoshi Shpitzen,[3] Atakan Aydin,[1] Ayeleth Reshef,[3] Magda Rosenthal,[1] Osnat Eliav,[3] Astrid Mühl,[1] Adam Lowe,[6] Danny Schurr,[3] Dror Harats,[7] Evi Jeschke,[1] Yechiel Friedlander,[4] Herbert Schuster,[1] Friedrich C. Luft,[1] and Eran Leitersdorf[3]

[1]Franz Volhard Clinic and Max Delbrück Center for Molecular Medicine and [2]Bernhard Nocht Institute for Tropical Medicine, Hamburg; [3]The Center for Research, Prevention, and Treatment of Atherosclerosis, Department of Medicine, Hadassah University Hospital, and [4]Department of Social Medicine, Hebrew University, Hadassah School of Public Health, Jerusalem; [5]Laboratory of Statistical Genetics, Rockefeller University, New York; [6]PE Biosystems, Foster City, CA; and [7]Institute of Lipid and Atherosclerosis Research, Sheba Medical Center, Tel-Hashomer, Israel

Summary

A cholesterol-lowering gene has been postulated from familial hypercholesterolemia (FH) families having heterozygous persons with normal LDL levels and homozygous individuals with LDL levels similar to those in persons with heterozygous FH. We studied such a family with FH that also had members without FH and with lower-than-normal LDL levels. We performed linkage analyses and identified a locus at 13q, defined by markers D13S156 and D13S158. FASTLINK and GENEHUNTER yielded LOD scores >5 and >4, respectively, whereas an affected-sib-pair analysis gave a peak multipoint LOD score of 4.8, corresponding to a *P* value of 1.26×10^{-6}. A multipoint quantitative-trait-locus (QTL) linkage analysis with maximum-likelihood binomial QTL verified this locus as a QTL for LDL levels. To test the relevance of this QTL in an independent normal population, we studied MZ and DZ twin subjects. An MZ-DZ comparison confirmed genetic variance with regard to lipid concentrations. We then performed an identity-by-descent linkage analysis on the DZ twins, with markers at the 13q locus. We found strong evidence for linkage at this locus with LDL ($P < .0002$), HDL ($P < .004$), total cholesterol ($P < .0002$), and body-mass index ($P < .0001$). These data provide support for the existence of a new gene influencing lipid concentrations in humans.

Received July 7, 1999; accepted October 1, 1999; electronically published January 4, 2000.

Address for reprints and correspondence: Dr. Friedrich C. Luft, Franz Volhard Clinic, Wiltberg Strasse 50, 13125 Berlin, Germany. E-mail: luft@fvk-berlin.de

* The first two authors contributed equally to this work.

0002-9297/2000/6601-0019$02.00

Introduction

Elevated serum LDL concentrations are a major cause of coronary atherosclerosis (Grundy 1997). Support for LDL's fundamental role derives from the discovery of the LDL receptor. Familial hypercholesterolemia (FH) is the most common (frequency 1/500) autosomal-dominant disease affecting lipid metabolism (Brown and Goldstein 1986); heterozygous affected persons have LDL levels twice normal levels and develop premature coronary disease, whereas homozygous individuals have sixfold-elevated LDL levels and often die of cardiovascular disease at age <20 years. Cholesterol-synthesis inhibitors have exerted a gratifying effect on the course of atherosclerosis (Gould et al. 1998). However, even more ingenious would be endogenous mechanisms that have a similar cholesterol-lowering effect. Hobbs et al. (1989) presented strong evidence supporting the notion that a "lipid-lowering" gene exists. They described a family with FH that featured affected members with lower-than-expected LDL concentrations. We recently identified a large Arab family with FH whose FH-affected family members often have normal LDL concentrations and do not manifest atherosclerosis. Thus far, 96 family members have been phenotyped and genotyped. The LDL-receptor mutation in this family has been identified elsewhere (Davis et al. 1986). The defect resides in the receptor's cytoplasmic tail and causes defective receptor internalization with almost-absent LDL-receptor activity. In the present investigation, we tested the hypothesis that a "cholesterol-lowering" gene exists in this family, by means of linkage analysis. We then studied a second, normal population and verified the relevance of our findings. We present evidence that such a gene indeed exists on chromosome 13q.

Methods

The FH family is Moslem and Arab and resides in Israel. Ninety-six members were examined after written

informed consent was obtained. Venous blood was used for DNA extraction and automated serum lipid measurements. LDL-cholesterol levels were calculated by use of the Friedewald formula (Friedewald et al. 1972). Repeated blood sampling and determinations of lipoprotein concentrations were done to ensure validity of the measurements prior to initiation of cholesterol-lowering therapy. At the time of the determinations, the subjects ingested a diet typical for the region, which is relatively high in fat and dairy products. We have subsequently advised persons with LDL-receptor mutations to adopt a low-fat diet and have begun pharmacological treatment as clinically indicated.

After we had determined the FH status (either heterozygous, homozygous, or not affected with FH) in all the family members, we examined the relationship between LDL cholesterol, age, gender, and body-mass index (BMI). We found no effects of gender and BMI in FH-heterozygous individuals; however, there was a modest age-related effect on LDL values in FH heterozygotes (see below). We also observed that the variation in LDL values increased with age. We then corrected the LDL-cholesterol values for age. The age-related effect on the variation was greater than the age-related effect on LDL values per se. We calculated the residuals of the LDL-cholesterol values by using standard linear regression, with age as an independent variable. This was done separately for FH-heterozygous affected and normal individuals in this family. We next corrected these residuals for age. This procedure allowed us to adjust the LDL values both for age and age-related variability. With these values we performed commingling analysis in the pedigree by using ILINK (Lathrop et al. 1984). These results allowed us to define FH-heterozygous persons with corrected LDL values $\leq$150 mg/dl as "affected" by a putative cholesterol-lowering gene (for justification, see the Results section). Those persons having LDL values >150 mg/dl were defined as not affected by this gene. We did not have sufficient FH-homozygous subjects to correct their LDL values for age. Those homozygous persons with an LDL-cholesterol concentration $\leq$500 mg/dl were classified as "affected" with the cholesterol-lowering gene, whereas those with LDL levels >500 mg/dl were classified as not affected. We picked these values of LDL concentrations because FH-heterozygous patients rarely have LDL concentrations >500 mg/dl and because <500 mg/dl in homozygous FH individuals are decidedly unusual. Moreover, such homozygous FH patients were asymptomatic in an earlier study (Sprecher et al. 1984). In persons carrying no FH mutation, there was a clear correlation between LDL values and age. LDL values were also higher in men than in women. Therefore, in this group the definition of "affected by the cholesterol-lowering gene" was more complex. We used the standardized linear-regression residuals of LDL versus age, done separately for men and women, to define persons as affected if LDL values were sharply ($\geq$1.5 SD) below the values predicted on the basis of their age and gender.

PCR-based mutation screening was performed in all 96 family members to categorize them as homozygous, heterozygous, or unaffected for FH. Genotyping was done with the ABI PRISM Genotyping System, including the Linkage Mapping Set, version 2 (LMS V2), PCR 9600 thermocyclers, the 877 Integrated Thermocycler, ABI DNA Sequencers, and GENESCAN and GENOTYPER software from PE Biosystems. The PCR primers contained in the LMS V2 amplify dinucleotide-repeat loci spaced at ~10 cM. The markers are organized in a set of 28 panels, with 10–19 primer pairs per panel, whose products can be electrophoresed and detected in a single lane. The LMS V2 set includes forward primers that are labeled with 6-FAM, HEX, and NED (replacing TET) fluorescent dyes. These dyes can be distinguished by their different spectral properties. The LMS V2 reverse primers were redesigned to overcome the problem of nontemplated nucleotide adenylation, also known as "plus-A." All markers were amplified under a common set of PCR conditions, by use of the True Allele PCR mix (PE Biosystems) containing Ampli*Taq* Gold DNA Polymerase and a final $MgCl_2$ concentration of 2.5 mM, with a 55°C annealing temperature. Electrophoresis and detection were done on an ABI 377 DNA Sequencer equipped with GENESCAN 2.1 software. Genotyping was performed with GENOTYPER 2.1 software. Genotypes were exported as a text file for subsequent linkage analysis.

We sought to determine whether any genes whose products are known to affect the interaction of LDL with its receptor cosegregate with the cholesterol-lowering phenotype. We looked for linkage between the cholesterol-lowering phenotype and the gene for the LDL receptor itself, as well as the genes for the receptor's two ligands, apo B-100 and apo E. Apo B gene mutations are known to cause low plasma-cholesterol levels (Collins et al. 1988). Apo B VNTR genotyping and apo E genotyping were done as described elsewhere (Hixon and Vernier 1990).

We selected 18 individuals for the first genotyping scan, to form a core pedigree. The selected group should contain a goodly number of closely related affected persons, which should minimize the danger of misspecifying the pedigree structure and should yield a maximum LOD score >3. The actual choice was made by visual inspection and by verification of the latter criterion by simulation analysis.

For total-genome scanning, we used FASTLINK (Cottingham et al. 1993), version 4.0, for the two-point analysis in the core pedigree. GENEHUNTER (Kruglyak et al. 1996) was used for multipoint analysis in split ver-

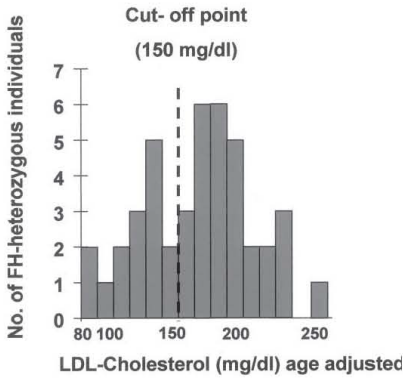


Figure 1 Frequency distribution of the subjects, in terms of their corrected LDL-cholesterol concentrations. Commingling analysis by use of ILINK allowed us to establish phenotypic criteria in terms of being "affected" by a putative cholesterol-lowering gene (LDL cholesterol <150 mg/dl).

sions of both the core and extended pedigrees. GENEHUNTER is not able to accommodate the multiple loops in the extended pedigree unless the pedigree is split into subunits. We therefore also elected to perform an affected-sib-pair analysis by using a maximum-likelihood binomial (MLB) statistic as implemented in a modified version of GENEHUNTER (Abel and Müller-Myhsok 1998). The MLB statistic is a maximum-likelihood ratio–based sibship method especially geared to the analysis of sibships with multiple affected persons. The analysis is based on the estimation of a single parameter, α, which is related to p, the proportion of alleles shared by the sib pair, according to the relationship $p = 1 - 2\alpha(1 - \alpha)$. The model used for parametric analysis was an autosomal-recessive–type model with an allele frequency of .01. We scrutinized all regions with a threshold p value of .1. Within the regions of interest, all 86 informative family members were genotyped. Because of computational restrictions, parametric multipoint linkage analysis within this highly consanguineous family was possible only by use of two flanking markers. When the results of the MLB analysis are considered, it should be noted that the LOD score obtained when this analysis is performed may be readily transformed into a χ^2 variable, by multiplying it by $2 \times \ln(10)$. This χ^2 variable asymptotically follows a 50:50 distribution of 0 and 1 df.

We also subjected our total genome–scan data to a test for linkage in terms of a quantitative-trait locus (QTL), using the novel QTL approach as implemented in MLBQTL (Alcais and Abel 1999). We chose this analysis because, contrary to the classically used Haseman-Elston approach, the MLBQTL does not necessarily assume a normal distribution of the trait or derived measures. For the analysis, we considered the phenotypic information from FH-pedigree members, who, after correction for gender, status at the FH locus, age, and the known covariate BMI, were ≥ 1 SD above or below the mean. Thus, we applied a combined discordant and concordant sib-pair QTL analysis.

Finally, to test the hypothesis that the cholesterol-modifying gene is present in the general population, we performed studies in 122 pairs of MZ and 100 pairs of DZ twins and in available parents of the DZ twins. The subjects were all healthy, normotensive white individuals recruited from various parts of Germany. The protocol was approved by Humboldt University's committee on the protection of human subjects, and written informed consent was obtained from all participants. Persons with histories of familial lipid disorders were excluded. Details of our twin analysis have been published elsewhere (Knoblauch et al. 1997). Blood was obtained for total cholesterol, HDL, and triglycerides, and LDL was calculated by use of the Friedewald equation (Friedewald et al. 1972). Blood was also obtained for the determination of zygosity and other molecular-genetic studies. Microsatellite markers D13S1306, D13S170, D13S1241, D13S265, D13S159, and D13S158, spanning the "cholesterol-lowering gene" locus on chromosome 13, were analyzed.

Table 1

Demographic Information on Individuals in the Core Pedigree

Individual	Age[a] (Sex)	LDL (age and FH adjusted)[b] (mg/dl)	FH	Apo Genotype ApoB	Apo Genotype ApoE[c]
IV:1	62 years (F)	341 (238)	1	1,2	3,3
IV:2	63 years (M)	111* (126*)	1	1,3	3,3
IV:4	57 years (F)	71* (104*)	1	3,3	3,3
IV:5	54 years (M)	135* (137*)	1	1,3	3,4
IV:6	55 years (M)	190 (167)	1	1,3	3,4
IV:7	55 years (F)	223 (185)	1	1,4	3,3
IV:8	60 years (F)	221 (181)	1	1,2	3,3
IV:9	65 years (M)	159 (ND)	0	3,3	3,3
V:1	42 years (F)	140* (140*)	1	2,3	3,3
V:3	35 years (F)	165 (159)	1	2,3	ND
V:5	... (F)	207 (...)	1	1,1	3,4
V:6	24 years (F)	136* (136*)	1	1,1	3,3
V:7	32 years (M)	211 (197)	1	1,1	3,4
V:8	32 years (F)	187 (178)	1	2,3	3,3
VI:1	3 years (F)	142* (143*)	1	2,2	3,3
VI:2	7 years (M)	111* (85*)	1	1,3	3,3
VI:3	5 years (M)	126* (110*)	1	1,3	3,3
VI:4	3 years (M)	115* (80*)	1	1,2	3,4

[a] An ellipsis (...) denotes that the individual's data were unknown.
[b] Values for individuals with the putative cholesterol-lowering gene are denoted by an asterisk (*). ND = not done.
[c] ND = not done.

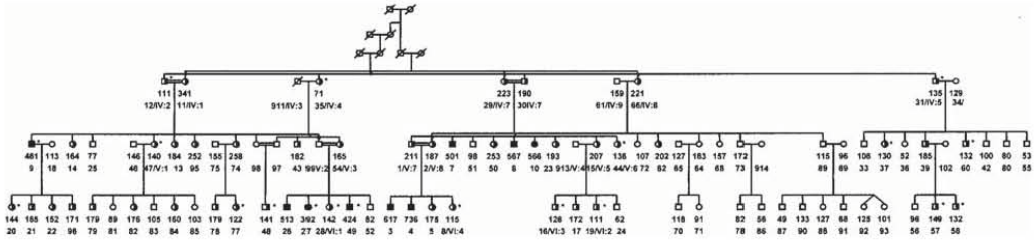


Figure 2 Extended pedigree showing all 96 individuals from whom lipid measurements were obtained. The subject's pedigree identification number is given, as well as the actual LDL-cholesterol concentration (mg/dl). FH-heterozygous individuals are half-blackened, whereas homozygous persons are fully blackened. Gray symbols represent those individuals selected for the core pedigree. Individuals with FH bearing the cholesterol-lowering gene are denoted by an asterisk (*). Their LDL-cholesterol levels are decidedly lower than would be expected for patients with FH.

For the twin linkage analysis, DZ pairs and their parents were included as described elsewhere (Busjahn et al. 1999). Analysis was done by use of a variance-component approach (Eaves et al. 1996). Phenotypic variance was decomposed into variance due to genetic background (A), variance due to the QTL effect (Q), and environmental variance (E): $\text{Var} = A^2 + Q^2 + E^2$. For the three possible identical by descent (IBD) states (sharing zero, one, or two alleles), covariance of a sib pair was then defined by $\text{Cov}_{\text{IBD0}} = 0.5A^2$, $\text{Cov}_{\text{IBD1}} = 0.5A^2 + 0.5Q^2$, and $\text{Cov}_{\text{IBD2}} = 0.5A^2 + Q^2$. To improve estimates of total variance and genetic background, MZ twins were included in the analysis, with the covariance defined as $\text{Cov}_{\text{MZ}} = A^2 + Q^2$.

To test for a QTL effect, the difference in model fit for models with and without a QTL effect was calculated as a χ^2 statistic. For each sib pair and each locus, the proportion of alleles IBD, based on parental genotypes and independent allele-frequency estimates, was calculated by use of a multipoint approach, as implemented in MAPMAKER/SIBS (Kruglyak and Lander 1995). The higher power of the variance-covariance–based analysis, compared with the squared trait differences–based approach by the Haseman-Elston method, has been shown in a recent simulation study (Fulker and Cherny 1996). Because we used a candidate-gene approach, we accepted $P < .01$ to test for significant linkage, in accordance with the criteria defined by Lander and Kruglyak (1995).

Heritability was estimated by structural equation modeling (Neale and Cardon 1992, p. 496), by use of the MX program developed by Neale (1997). The variability of any given phenotype within a population can be decomposed into genetic influences (A), environmental influences shared by the twins within a family (C), and effects of random environment (E): $\text{Var} = A^2 + C^2 + E^2$. For MZ and DZ, the covariance of their phenotype is given by $\text{Cov}_{\text{MZ}} = A^2 + C^2$ and $\text{Cov}_{\text{DZ}} = 0.5A^2 + C^2$. Genetic, as well as environmental, effects were estimated by the best-fitting model as selected by the χ^2 value. Statistical analysis was conducted by use of the SPSS program. Adjustment of phenotypic values for sex and age was done by multiple linear regression, with the unstandardized residuals as the corrected phenotypes. In case of significant deviations from a normal distribution, the appropriate transformations were applied prior to analysis.

Results

In the FH-heterozygous family members, we found a modest, albeit significant ($P < .04$), effect of age on LDL values, allowing us to correct for the effects of age. Figure 1 shows the frequency distribution of corrected LDL-cholesterol values in FH-heterozygous subjects. Commingling analysis using ILINK gave significant evidence ($P < .03$) against a unimodal distribution of the corrected LDL values. The mean LDL value of the lowest genotypic distribution in the model was 138 mg/dl; the SD was 29 mg/dl. The other means were close together, at 198 and 203 mg/dl, respectively. This finding was interpreted as indicating a recessive trait, which was in accordance with a segregation analysis that used LOKI (Heath 1997). The classification threshold (150 mg/dl) was calculated as the 5th percentile in the group of persons heterozygous for the putative cholesterol-lowering gene. This approach gave a 95% certainty of correct classification for heterozygous individuals. We classified FH-heterozygous persons with values ≤150 mg/dl as affected by a cholesterol-lowering trait. The extended pedigree is shown in figure 2. FH-heterozygous individuals are indicated, as well as the uncorrected LDL-cholesterol values. Persons affected by the cholesterol-lowering gene are also indicated.

Table 2

Two-Point LOD Scores in the Core Pedigree

	LOD Score at θ =						
Marker	0	.01	.05	.1	.2	.3	.4
D13S175	−∞	−.48	.32	.56	.55	.36	.17
D13S217	−∞	−5.82	−2.75	−1.46	−.42	−.07	.02
D13S171	−∞	−4.15	−1.72	−.74	−.05	.1	.09
D13S263	−∞	−7.04	−2.95	−1.38	−.19	.15	.16
D13S153	−∞	−7.06	−2.92	−1.35	−.18	.14	.15
D13S156	−∞	−2.2	−.37	.35	.74	.58	.27
D13S1306	.55	.67	.85	.84	.62	.34	.12
D13S789	−∞	−2.84	−1.18	−.4	.16	.25	.16
D13S170	−∞	1.94	2.29	2.14	1.53	.83	.26
D13S271	−∞	.13	1.17	1.38	1.19	.77	.33
D13S265	−∞	.67	1.56	1.71	1.44	.92	.39
D13S794	−∞	−1	.68	1.09	1.03	.62	.21
D13S795	−∞	−1.93	−.54	0	.28	.22	.1
D13S1300	.54	.58	.88	1	.81	.46	.16
D13S129	3.67	3.69	3.56	3.2	2.25	1.21	.39
D13S125	−.29	−.09	.4	.58	.52	.29	.09
D13S254	−∞	3.06	3.32	3.06	2.23	1.29	.49
D13S154	−∞	−1.2	.49	1	1	.56	.14
D13S1241	−∞	1.67	2.25	2.22	1.67	.92	.29
D13S786	−∞	−2.54	−.41	.33	.67	.54	.26
D13S159	−∞	−1.82	.19	.81	.95	.67	.3
D13S158	−∞	−2.16	−.22	.43	.63	.39	.12
D13S173	−∞	−1.97	−.65	−.18	.08	.09	.05
D13S285	−∞	−5.04	−2.39	−1.22	−.31	−.03	.02

We first looked for linkage between the cholesterol-lowering–gene locus and the gene for the LDL receptor itself, as well as the genes for the receptor's two ligands, apo B-100 and apo E. No significant evidence for linkage was found with either of these candidates. We next constructed a core pedigree consisting of 29 individuals composed of normal and FH-heterozygous individuals only. A subset of 18 individuals from this core pedigree (table 1) was genotyped for 300 microsatellite markers. Markers on eight chromosomal regions (two regions each on chromosomes 1 and 12 and one region each on chromosomes 6, 13, 16, and 18) were candidates ($P <$ .1) for linkage to the low-cholesterol phenotype.

Eighty-six individuals from the 96-member pedigree were then used to test for linkage with additional markers from the eight chromosomal regions. Ten persons were not included because they were not informative for the linkage analysis. In the core pedigree, a maximum pairwise LOD score of 3.69 was obtained with marker D13S129, at a recombination fraction (θ) of .01. Also in the core pedigree, marker D13S254 yielded a maximum pairwise LOD score of 3.32 at θ = .05 (table 2). The maximum two-point LOD score in the entire pedigree was 5.22 with marker D13S129 at θ = .05. Two additional markers exceeding the critical threshold of 3.0 were D13S265, with a LOD score of 4.06 at θ = .10, and D13S1241, with a LOD of 3.01 at θ = .10. We also analyzed the data set as an "affecteds-only" analysis (table 3). Multipoint linkage analysis in the extended pedigree was hampered by the complex consanguinity and by possible additional, unrecognized interconnections. Nevertheless, a three-point analysis using FASTLINK gave a LOD score of 5.67 at a location close to D13S129. GENEHUNTER yielded a maximum multipoint LOD score of 4.50 at θ = .0, at the neighboring marker D13S1300, which flanks D13S129. To circumvent potential limitations of FASTLINK and GENEHUNTER, we performed the affected-sib-pair analysis with the MLB statistic. Significant positive LOD scores were obtained exclusively for markers from the same region on chromosome 13, as shown in figure 3. The maximum multipoint LOD score (when the MLB statistic was used) was 4.82 (corresponding to a nominal P value of 1.26×10^{-6}) at D13S1241. This region demonstrated complete sharing among affected sibs. The critical region on the basis of the "z_{max}-2" method for this analysis, was defined by two polymorphic markers, D13S156 and D13S158, which are ~37 cM apart. We next verified our findings by showing independently that the 13q locus is a QTL for LDL concentrations in this pedigree. QTL analysis using MLBQTL gave a maximum LOD of 4.23, with the peak located at D13S794 and with the "z_{max}-2" support interval also extending from D13S156 to D13S158. No other significant peaks

Table 3

Two-Point LOD Scores in the Whole Pedigree (Affecteds Only)

	LOD Score at θ =						
Marker	0	.01	.05	.1	.2	.3	.4
D13S175	−.41	−.35	−.17	−.06	.02	.03	.01
D13S217	−1.68	−1.54	−1.12	−.78	−.39	−.18	−.07
D13S171	−1.64	−1.51	−1.11	−.79	−.41	−.2	−.07
D13S263	−1.63	−1.42	−.89	−.51	−.15	−.01	.03
D13S153	−2.84	−2.52	−1.67	−1.03	−.37	−.08	.01
D13S156	−.12	.03	.32	.42	.36	.2	.07
D13S1306	.9	.89	.82	.71	.46	.23	.07
D13S789	.69	.67	.6	.5	.33	.18	.07
D13S170	1.63	1.63	1.51	1.29	.78	.36	.09
D13S271	1.59	1.54	1.34	1.11	.68	.34	.11
D13S265	2.37	2.3	2	1.65	1.04	.55	.19
D13S794	1.34	1.3	1.15	.96	.58	.27	.07
D13S795	.58	.57	.53	.47	.31	.15	.04
D13S1300	1.78	1.72	1.45	1.14	.62	.26	.06
D13S129	1.96	1.9	1.67	1.36	.8	.38	.11
D13S125	.68	.66	.59	.51	.35	.2	.08
D13S254	1.48	1.49	1.42	1.25	.83	.44	.15
D13S154	.72	.71	.65	.54	.31	.12	.01
D13S1241	.02	.14	.38	.45	.33	.15	.02
D13S786	.71	.77	.86	.82	.6	.33	.12
D13S159	.44	.56	.78	.81	.64	.37	.14
D13S158	−1.15	−1	−.63	−.39	−.18	−.09	−.05
D13S173	.46	.44	.38	.31	.19	.09	.02
D13S285	−.5	−.43	−.25	−.11	−.01	−.01	−−.02

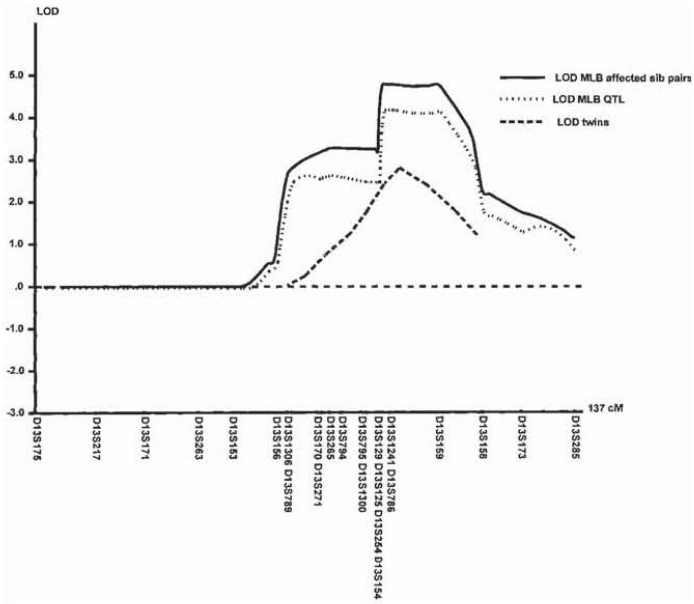


Figure 3 Results of linkage analysis using MLB and MLBQTL in the FH pedigree, together with linkage results for LDL in the DZ twins (*P* values have been transformed into LOD scores). In the twins, the peak level of significance was .0002, right on marker D13S1241.

(LOD score >3) in the genome were identified by use of the QTL analysis. One region on chromosome 6 (around D6S257) showed a LOD score of 1.95. Haplotype data show the cosegregation of the 13q markers with the phenotype, as shown in figure 4. The gray-shaded zone represents the area of complete allele sharing within all affected sib pairs. Nevertheless, there was no common region of homozygosity in all affected pedigree members. Two persons unaffected by the cholesterol-lowering trait have the same haplotypes as their affected siblings. These two individuals may represent incomplete penetrance.

To test the relevance of our findings, we performed a twin analysis in German subjects (table 4). Females were twice as common as males. The subjects were young adults of normal height, weight, and BMI. Total cholesterol, HDL, LDL, and triglyceride values were all within normal limits. Table 4 also shows the results of the heritability analysis. A major genetic effect was demonstrated for all lipid parameters, although strong environmental effects were also shown. Table 5 shows the results of the linkage analysis. Significant linkage was shown for HDL, LDL, total-cholesterol concentrations, and BMI. Figure 3 gives the results of the variance-component multipoint linkage analysis for LDL. The peak level of significance was .0002, right on marker D13S1241. For this locus, the lower boundary-effect estimates, on a 95% confidence interval, for LDL cholesterol and BMI were ~26% and ~22%, respectively.

Discussion

The important finding in our study is that we were able to identify significant linkage between a locus on chromosome 13q and a putative cholesterol-lowering gene in a family with FH that had members with unexpectedly low LDL concentrations. To solidify our results, we used more than one linkage analysis, including parametric linkage analysis, multipoint linkage analysis, an affected-sib-pair method, and a QTL analysis. At first glance, there appears to be a discrepancy between the parametric analysis and the affected-sib-pair analysis. The peak LOD score from MLB and QTLMLB analysis is located near D13S786. This marker and flanking markers yielded a LOD score of $-\infty$ in the two-point linkage analysis. This result may be due to uncertainties in the genetic models used in the analysis. More specifically, these problems may reside in the penetrance matrix. None of these recombination events was present in an affected individual. This fact is shown by the table of LOD scores from the affecteds-only analysis in the

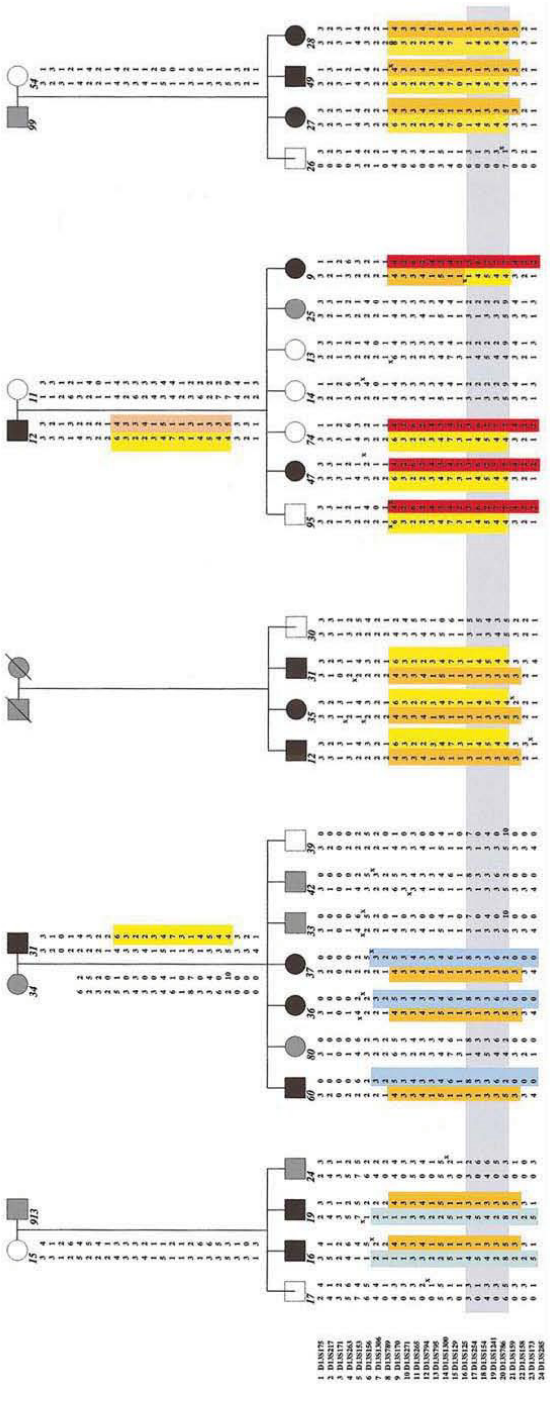


Figure 4 Nuclear pedigrees selected for sib-pair analysis, shown in terms of the cholesterol-lowering trait. Unblackened and blackened symbols represent unaffected and affected individuals, respectively; gray symbols represent unknown status. Haplotypes from the candidate region on chromosome 13 are shown below the individuals. Identical haplotypes are marked in the same color. The smallest area of complete sharing among all sibs is indicated by the horizontal gray bar.

Table 4

Clinical Data and Serum Lipid Values for the German Twin Sample

Variable	MZ Twins	DZ Twins	Genetic Effect	*P*
n (pairs)	122	100		
Age (years)	34 ± 15	34 ± 13		
Sex (male/female)	80/164	60/140		
Height (cm)	169 ± 9	170 ± 9		
Weight (kg)	67 ± 13	71 ± 14		
BMI (kg/m^2)	23 ± 4	24 ± 4	.97	.01
Total cholesterol (mg/dl)	183 ± 39	193 ± 42	.64	.01
HDL cholesterol (mg/dl)	51 ± 14	57 ± 17	.59	.01
LDL cholesterol (mg/dl)	115 ± 34	115 ± 32	.66	.01
Triglycerides (mg/dl)	87 ± 67	100 ± 64	.72	.01

NOTE.—Data are mean ± SD unless otherwise indicated.

entire pedigree (table 3). We observed a series of 10 markers (D13S1306–D13S254) showing positive LOD scores, with $\theta = 0$ in every case.

We interpreted the lack of a common homozygosity region in this family as being due to a high frequency of this modifier gene in the general population. A rough estimate suggests a population allele frequency of .10, which is also the value used for the linkage analysis. Furthermore, the family that we studied is from an isolated but stable population. The expected region of homozygosity may be detectable only at the intragenic level, as has been described by others (Aksentijevich et al. 1999). Aksentijevich et al. (1999) performed haplotype studies of familial Mediterranean fever in the Ashkenazi Jewish population. There were two completely different haplotypes (for six markers) in that study, both carrying the sequence alteration V726A in the same family. Similarly, Aksentijevich et al. (1999) point out that the mutation E148Q may extend back to a common relatively ancient founder. Several distinct haplotypes carrying this mutation have been observed. However, at the intragenic level, the single nucleotide–polymorphism motifs converged. Those investigators concluded that an ancestor common to all the E148Q carriers may have lived some 1,500–2,000 years ago, a time span not unrealistic for this part of the world. For these reasons, we believe that our failure to identify a homozygosity region does not detract from the significance of our findings. Another possible explanation for our findings is related to the frequency of the mutation. A commonly occurring mutation could very well enter through different pathways even in a sibship, especially through affected parents (Veske et al. 1996). There are several examples of such sibships in our study.

To test whether this new locus is of any relevance to the general population, we performed a second, independent study in MZ and DZ twins. We verified earlier findings that LDL, HDL, total cholesterol, triglycerides, and BMI are all strongly influenced by genetic variance (Knoblauch et al. 1997). An IBD linkage analysis in DZ twins yielded strong evidence in support of the 13q locus as being a QTL for lipid concentrations, as well as for BMI. Moreover, the effect of this locus on LDL cholesterol was estimated to be ~26%. We believe that these studies are unique, since we not only mapped a heretofore unknown gene but also proved, in principle, its effects and relevance in a completely unrelated, normal population.

Our results extend earlier studies indicating the existence of a cholesterol-lowering gene. Hobbs et al. (1989) described a similar unusual kindred with FH, in which one-third of affected relatives had normal LDL levels. Those investigators concluded that the low-cholesterol phenotype was most consistent with a single dominant gene suppressing the hypercholesterolemic effect of the LDL-receptor mutation in the family that they studied. They also excluded the LDL-receptor gene and the apo B-100 and apo E genes, by linkage analysis. Nora et al. (1985) also described a family with FH with normal cholesterol values in obligate-heterozygous patients with FH. Conceivably, by expansion of these pedigrees, a linkage analysis to verify and extend our results could be performed.

To test for linkage, we first performed an affecteds-only analysis on the core pedigree, which yielded a promising LOD score of 2.31 at D13S170. We next performed a parametric two-point linkage analysis in the core pedigree, assuming complete penetrance and including unaffected individuals according to our phenotypic definitions, which yielded two closely neighboring markers with LOD scores >3 at markers D13S254 and D13S129. We then examined the extended pedigree according to a conventional, parametric, two-point-linkage technique, which yielded a LOD score of 5.22 at marker D13S129. Three-point analysis using FASTLINK gave a LOD score of 5.67 at a location close to D13S129. A GENEHUNTER analysis gave a LOD score of 4.50 in the area of this same marker. An affected-sib-pair approach using the MLB statistic provided a LOD score of 4.82, corresponding to a nominal *P* value of 1.25×10^{-6}. We believe that our approach, using sev-

Table 5

Results of Linkage Analysis

Model	χ^2 Model Difference (*P*)[a]
Total cholesterol	13.63 (.0002)
HDL cholesterol	8.20 (.004)
LDL cholesterol	13.60 (.0002)
Triglycerides	6.39 (.011)
BMI	14.26 (.0001)

[a] The difference is between models with and without a QTL effect; *P* values are for the corresponding QTL effect.

eral methods, provides robust evidence that the cholesterol-lowering gene resides on chromosome 13q.

Since LDL values are a continuous trait, our definition of being affected or unaffected by the cholesterol-lowering gene is potentially arbitrary until we identify the responsible gene mutation. We took care to consider the effects of age, gender, and BMI on LDL values. Our affected cutoff point is in accord with the accepted definition of FH among close relatives, for persons age >18 years, which is 165 mg/dl (Williams et al. 1996). Additional support in favor of our phenotypic definition comes from the analysis of corrected LDL concentrations of Druze and Christian-Lebanese FH-heterozygous individuals, who consume a diet very similar to that of the Moslem-Arab individuals whom we studied. The FH-heterozygous persons defined as affected for the cholesterol-lowering gene in our study had corrected LDL concentrations within the range seen in persons without FH who were from the Druze and Christian-Lebanese families with FH that have been described elsewhere (Leitersdorf et al. 1991). In contrast to the situation for individuals with FH in China (Sun et al. 1994) and Tunisia (Slimane et al. 1993), who, because of the ingestion of a low-fat diet, can have similarly low LDL values, we can rule out any dietary effect. The diet of the family with FH examined by us does not differ from the usually high-fat diet ingested by other people from this area. Their diet is no different from that ingested by the Christian-Arab population residing in Lebanon and described initially by Khachadurian (1964). Interestingly, Khachadurian observed that, with the exception of four FH-homozygous patients from a single family, 50 other FH-homozygous patients all had cholesterol values >600 mg/dl. The families that he studied had a null mutation similar to the mutation in the family that we studied, which results in an almost completely nonfunctioning LDL receptor. Finally, in the family that we studied, lipid values from persons not affected by the putative cholesterol-lowering gene are in the expected range, as in other families with FH in Israel.

The potential arbitrariness of our phenotype—and the clue that the allele in question might be common—led us first to demonstrate that the 13q locus is indeed a QTL in the pedigree in question and then to prove that the 13q locus has relevance to lipid concentrations in the general population. Relevance for the general population can usually not be shown for monogenic diseases. FH, for example, is caused by literally hundreds of separate mutations in the LDL-receptor gene (Hobbs et al. 1992). However, linkage of the LDL receptor–gene locus to LDL serum concentrations in normal subjects or in patients without FH and with coronary disease has not been convincingly shown (Knoblauch et al. 1997).

DZ twins are a particularly powerful sib-pair model, because of identical ages and a shared environment, at least during childhood. Sample size can be sharply reduced, without a loss of power, when DZ-twin siblings are examined. The utility of DZ twins in the quantitative sib-pair linkage–analysis approach to genes relevant to cardiovascular disease was recently demonstrated by Austin et al. (1998), who found linkage between the microsomal triglyceride-transfer–protein gene locus and plasma triglyceride concentrations. In addition, our earlier twin study (Knoblauch et al. 1997) showed evidence for linkage between the macrophage-scavenger–receptor gene locus and HDL concentrations. Our linkage results are almost two orders of magnitude below the criteria suggested by Lander and Kruglyak (1995), who, for a successful replication of an earlier result, called for a P value of $\leq .01$.

We have yet to identify potentially interesting candidate genes in this chromosomal area. As part of an effort to dissect the genetic factors involved in cholesterol homeostasis, a genomewide analysis with a mouse model revealed several new candidate genes (Welch et al. 1996). However, none of these loci are syntenic to the homologous human chromosome (13q). We believe that low cholesterol levels in the affected individuals in this pedigree may be caused by a single gene. Although this hypothesis is speculative, we suggest that this gene may act on lipoprotein assembly in the liver. Functional studies will be required in order to test this issue. Elucidation of this gene may give new insight into mechanisms of lipoprotein regulation and protection from atherosclerosis and could lead to a new class of cholesterol-lowering agents.

Acknowledgments

Support was provided by a grant-in-aid from the Sarah and Moshe Mayer Foundation for Research (to E.L.) and support from PE Biosystems and LION Bioscience AG (to H.S.).

References

Abel L, Müller-Myhsok B (1998) Robustness and power of the maximum likelihood binomial (MLB) and the maximum likelihood score (MLS) methods in multipoint linkage analysis of affected sibship data. Am J Hum Genet 63:638–647

Aksentijevich I, Torosyan Y, Samuels J, Centola M, Pras E, Chae JJ, Oddoux C, et al (1999) Mutation and haplotype studies of familial Mediterranean fever reveal new ancestral relationships and evidence for a high carrier frequency with reduced penetrance in the Ashkenazi Jewish population. Am J Hum Genet 64:949–962

Alcais A, Abel L (1999) Maximum-Likelihood-Binomial method for genetic model-free linkage analysis of quantitative traits in sibships. Genet Epidemiol 17:102–117

Austin MA, Talmud PJ, Luong L-A, Haddad L, Day INM, Newman B, Edwards LK, et al (1998) Candidate-gene studies of the atherogenic lipoprotein phenotype: a sib-pair link-

age analysis of DZ women twins. Am J Hum Genet 62: 406–419

Brown MS, Goldstein JL (1986) A receptor-mediated pathway for cholesterol homoestasis. Science 232:34–47

Busjahn A, Knoblauch H, Faulhaber H-D, Uhlmann R, Hoehe M, Schuster H, Luft FC (1999) The QT interval is linked to two long-QT syndrome loci in normal subjects. Circulation 99:3161–3164

Collins DR, Knott TJ, Pease RJ, Powell LM, Wallis SC, Robertson S, Pullinger CR, et al (1988) Truncated variants of apolipoprotein B cause hypobetalipoproteinemia. Nucleic Acids Res 16:8361–8375

Cottingham RW Jr, Idury RM, Schäffer AA (1993) Faster sequential genetic linkage computations. Am J Hum Genet 53: 252–263

Davis CG, Lehrman MA, Russell DW, Anderson RG, Brown MS, Goldstein JL (1986) The J.D. mutation in familial hypercholesterolemia: amino acid substitution in cytoplasmic domain impedes internalization of LDL receptors. Cell 45: 15–24

Eaves LJ, Neale MC, Maes H (1996) Multivariate multipoint linkage analysis of quantitative trait loci. Behav Genet 26: 519–525

Friedewald WT, Levy RI, Fredrickson DS (1972) Friedewald formula: estimation of the concentration of low density lipoprotein cholesterol in plasma without the use of preparative ultracentrifugation. Clin Chem 18:499–502

Fulker DW, Cherny SS (1996) An improved multipoint sib-pair analysis of quantitative traits. Behav Genet 26:527–532

Gould AL, Rossouw JE, Santanello NC, Heyse JF, Furberg CD (1998) Cholesterol reduction yields clinical benefit: impact of statin trials. Circulation 97:946–952

Grundy SM (1997) Cholesterol and coronary heart disease. Arch Intern Med 157:1177–1184

Heath S (1997) Markov chain Monte Carlo segregation and linkage analysis for oligogenic models. Am J Hum Genet 61:748–760

Hixon JE, Vernier DT (1990) Apo E genotyping: restriction isotyping of human apolipoprotein E by gene amplification and cleavage with *Hha*I. J Lipid Res 31:545–548

Hobbs HH, Brown MS, Goldstein JL (1992) Molecular genetics of the LDL receptor gene in familial hypercholesterolemia. Hum Mutat 1:445–466

Hobbs HH, Leitersdorf E, Leffert CC, Cryer DR, Brown MS, Goldstein JL (1989) Evidence for a dominant gene that suppresses hypercholesterolemia in a family with defective low density lipoprotein receptors. J Clin Invest 84:656–664

Khachadurian AK (1964) The inheritance of essential familial hypercholesterolemia. Am J Med 37:402–410

Knoblauch H, Busjahn A, Münter S, Nagy Z, Faulhaber H-D, Schuster H, Luft FC (1997) Heritability analysis of lipids and three gene loci in twins link the macrophage scavenger receptor to high-density lipoprotein cholesterol concentrations. Arterioscler Thromb Vasc Biol 17:2054–2060

Kruglyak L, Daly MJ, Reeve-Daly MP, Lander ES (1996) Parametric and nonparametric linkage analysis: a unified multipoint approach. Am J Hum Genet 58:1347–1363

Kruglyak L, Lander ES (1995) Complete multipoint sib-pair analysis of qualitative and quantitative traits. Am J Hum Genet 57:439–454

Lander ES, Kruglyak L (1995) Genetic dissection of complex traits: guidelines for interpreting and reporting linkage results. Nat Genet 11:241–246

Lathrop GM, Lalouel JM, Julier C, Ott J (1984) Strategies for multilocus linkage analysis in humans. Proc Natl Acad Sci USA 81:3443–3446

Leitersdorf E, Friedlander Y, Bard JM, Fruchart JC, Eisenberg S, Stein JC (1991) Diverse effects of ethnicity on plasma lipoprotein (a) levels in heterozygous patients with familial hypercholesterolemia. J Lipid Res 32:1513–1519

Neale MC (1997) Mx: statistical modeling, 4th ed. Virginia Commonwealth University, Richmond

Neale MC, Cardon LR (1992) Methodology for genetic studies of twins and families. Kluwer Academic, Dordrecht, the Netherlands

Nora JJ, Lortscher RM, Spangler RD, Bilheimer DW (1985) I. Familial hypercholesterolemia with "normal" cholesterol in obligate heterozygotes. Am J Med Genet 22:585–591

Slimane MN, Pousse H, Maatoug F, Hammami M, Ben-Farhat MH (1993) Phenotypic expression of familial hypercholesterolemia in central and southern Tunisia. Atherosclerosis 104:153–158

Sprecher DL, Schaefer EJ, Kent KM, Gregg RE, Zech LA, Hoeg MM, McManus B, et al (1984) Cardiovascular features of homozygous familial hypercholesterolemia: analysis of 16 patients. Am J Cardiol 54:20–30

Sun XM, Patel DD, Webb JC, Knight BL, Fan LM, Cai HJ, Soutar AK (1994) Familial hypercholesterolemia in China: identification of mutations in the LDL-receptor gene that result in a receptor-negative phenotype. Arterioscler Thromb 14:85–94

Veske A, Oehlmann R, Younus F, Mohyuddin A, Müller-Myhsok B, Mehdi SQ, Gal A (1996) Autosomal recessive nonsyndromic deafness locus maps to chromosome 21q22 in a large consanguineous kindred from Pakistan. Hum Mol Genet 5:165–168

Welch CL, Xia YR, Shechter I, Farese R, Mehrabian M, Mehdizadeh S, Warden CH, et al (1996) Genetic regulation of cholesterol homeostasis: chromosomal organization of candidate genes. J Lipid Res 37:1406–1421

Williams RR, Hamilton-Craig I, Kostner GM, Hegele RAA, Hayden MR, Pimstone SN, Faergeman O, et al (1996) MED-PED: an integrated genetic strategy for preventing early deaths: genetic approaches to noncommunicable diseases. Springer-Verlag, Berlin and Heidelberg

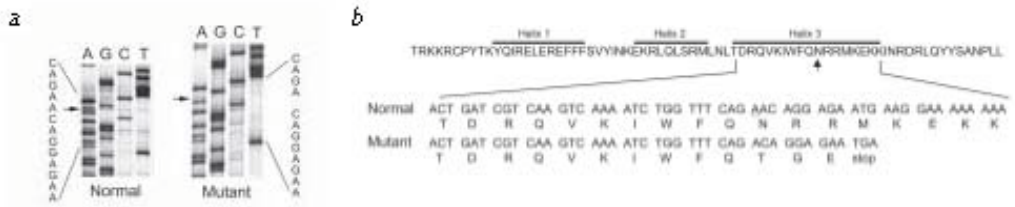


Fig. 2 Molecular analysis of *HOXA11*. *a*, Sequencing autoradiograph from an affected individual (mutant) shows a single nucleotide deletion (arrow) in exon 2 compared with the normal control. *b*, Amino acid sequence encoded by exon 2 of *HOXA11* demonstrates the location of the mutation and expected consequences. This region encodes the trihelical homeodomain. The point mutation identified in our patients occurs at a highly conserved asparagine residue (arrow) in the third helix, a region that is vital for DNA binding. Compared with normal sequence, this deletion (nucleotide underlined) results in premature termination codon and truncation of the remaining 22 aa of the HOXA11 protein.

human non-neoplastic haematologic disorder, and only the third HOX gene implicated in a human syndrome[6,7]. Although current data indicate a correlation between the point mutation and abnormal skeletal development, evidence positively linking the two is required. Studies of Hox genes have largely involved the use of null mutants, involving loss of function rather than an alteration of function. Mouse models of hypodactyly (*Hd*) and synpolydactyly (*spdh*), carrying mutations of *Hoxa13* and *Hoxd13*, respectively, reinforce the usefulness of examining non-null mutants to further our understanding of homeobox regulation[14,15]. Studies of this new *HOXA11* mutation will advance our knowledge of Hox gene function, and determine their roles in bone morphogenesis and early haematopoietic lineage commitment and proliferation.

Alexis A. Thompson & Long T. Nguyen

Department of Pediatrics, Division of Hematology/Oncology, Gwynne Hazen Cherry Memorial Laboratories, University of California, Los Angeles School of Medicine, Los Angeles, California, USA. Correspondence should be addressed to A.A.T. (e-mail: athompson@ucla.edu).

1. Aiker, B.P. *J. Hand Surg. Am.* 17, 568–571 (1992).
2. Schroeder-Kurth, T.M., Auerbach, A.D. & Obe, G. (eds) *Fanconi Anemia: Clinical, Cytogenetic and Experimental Aspects* 284 (Springer, Berlin, 1989).
3. Hall, J.G. *J. Med. Genet.* 24, 79–88 (1987).
4. Mark, M., Ghyselinck, N.B. & Chambon, P. *Pediatr. Res.* 42, 421–429 (1997).
5. Lawrence, H.J., Sauvageau, G., Humphries, R.K. & Largman, C. *Stem Cells* 14, 281–291 (1996).
6. Mortlock, D.P. & Innis, J.W. *Nature Genet.* 15, 179–180 (1997).
7. Mortlock, D.P., Post, L.C. & Innis, J.W. *Nature Genet.* 13, 284–289 (1996).
8. Hall, J.G. *J. Med. Genet.* 24, 79–88 (1987).
9. Davis, A.P. & Capecchi, M.R. *Development* 122, 1175–1185 (1996).
10. Pöll, E.M. & Chambon, P. *Curr. Opin. Genet. Dev.* 7, 481–487 (1997).
11. Small, K.M. & Potter, S.S. *Genes Dev.* 7, 2318–2328 (1993).
12. Thorsteinsdottir, U. *et al. Mol. Cell. Biol.* 17, 495–505 (1997).
13. Evans, T. *Hematol. Oncol. Clin. North Am.* 11, 1115–1147 (1997).
14. Mortlock, D.P., Post, L.C. & Innis, J.W. *Nature Genet.* 13, 284–289 (1996).
15. Johnson, K.R. *et al. Hum. Mol. Genet.* 7, 1083–1088 (1998).

A region on chromosome 3 is linked to dizygotic twinning

A chromosome 3 region containing the gene (*PPARG*) encoding peroxisome proliferator activated receptor (PPARγ) may be related to dizygotic twinning. Linkage in 181 dizygotic twin pairs yielded a 6.93 lod score. The *PPARG* C→T substitution allele was far less common in monozygotic twins than in dizygotic twins. Dizygotic heterozygosity was less than expected and transmission disequilibrium test (TDT) analysis gave further evidence for association.

We initially studied 222 pairs of normal young monozygotic (122) and dizygotic (100 pairs) twins in an investigation of *PPARG* as a candidate quantitative trait locus for cholesterol levels[1]. We analysed *D3S1297*, *D3S1304*, *D3S3728*, *D3S3589*, *D3S1263*, *D3S2338* and *D3S1266*, as well as the silent C→T substitution. Heterozygosity for this polymorphism was much lower than expected, causing us to hypothesize that *PPARG* is linked to dizygotic twinning. A linkage analysis in dizygotic twins (phenotype: being a dizygotic twin) whose parents were available for analysis yielded a multipoint 3.49 lod score. Mean sharing of alleles (π) was 0.64 (expected 0.50). After approval, we recruited 116 nontwin sibpairs from Berlin, 50 unselected dizygotic twin pairs from Finland and 31 unselected dizygotic twin pairs from Poland. The nontwin sibpairs did not deviate from mendelian transmission. The 181 dizygotic twin pairs gave a 6.93 lod score (π=0.65; Fig. 1). The C→T substitution allele was lower in monozygotic twins (0.09) and parents (0.13) than in dizygotic twins (0.19). Genotype-based tests were significant for monozygotic compared with dizygotic twins (P<0.001) and for parents compared with dizygotic twins (P<0.05). Allele frequencies of dizygotic twin mothers and fathers were not different (0.15 versus 0.12). In dizygotic twins, the C→T substitution was not in Hardy–Weinberg equilibrium (CC, 72%; CT, 18%; TT, 10%); homozygosity was over-represented. In monozygotic twins and parents of dizygotic twins, Hardy–Weinberg proportions were maintained.

The Pro12Ala *PPARG* polymorphism in exon B obeyed Hardy–Weinberg equilibrium. We compared allelic frequencies between dizygotic twins, monozygotic twins and the general population for five randomly selected microsatellites and other biallelic polymorphisms. We found a significant difference in dizygotic twins compared with monozygotic twins and the general population for only the C→T polymorphism in *PPARG*. We next carried out TDT tests in dizygotic twins whose parents were informative for analysis. Haplotype analysis was significant when both *PPARG* polymorphisms were studied, even when only one sibling was selected randomly from each pair. Single polymorphism TDTs were not significant.

Our evidence implicating the chromosome 3 region is supported by four

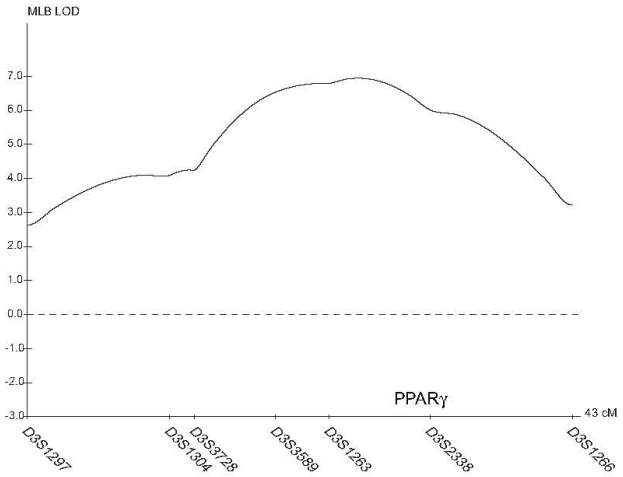


Fig. 1 The multipoint lod scores in all dizygotic twin subjects for the markers tested are shown, with *D3S3608* showing the maximum lod score of 6.93.

observations. We have evidence from model-free linkage analysis. We have a positive allelic association. Hardy–Weinberg proportions for the intragenic *PPARG* polymorphism were not maintained in dizygotic twins. We have evidence from a haplotype-based TDT analysis with both the biallelic Pro12Ala marker in exon B and the C→T substitution in exon 6. These two polymorphisms are not in tight linkage disequilibrium. Exon B is present in the *PPARG* splice variant PPARγ2, which is present in white fat, whereas exon 6 is present in both[2].

Multiple births require both multiple conceptions and intrauterine survival of both twins. 'Vanishing twins' are well known, with about 40% of spontaneous dizygotic twin pregnancies resulting in singleton births[3]. About 30% of all multiple pregnancies after assisted reproduction result in a singleton birth[4], and this effect is not explained by environmental factors[5]. Dizygous twinning has long been suspected to have a genetic basis[6]. Weinberg suggested that hereditary twinning is transmitted through the female line, applies only to dizygotic twins and is probably recessive[7]. In our study, the C→T substitution allele was greater in dizygotic twins compared with their mothers and fathers, and monozygotic twins, making a maternal effect less likely[8]. Evidence from Mormon records supports a recessive inheritance[9]. We found that the C→T polymorphism was not in Hardy–Weinberg equilibrium in dizygotic twins with an over-representation of homozygous individuals. This phenomenon has been described[10] as indicative of a heterogeneous recessive model.

A tenfold increase in the twinning rate has been recorded in a small village in southern Brazil[10]. High rates for twinning have also been recorded on the archipelago of Aland and Aboland, in southwest Finland[12]. Lummaa *et al.*[13] used Finnish church records in a case-control design and provided evidence that differences in twinning frequencies in isolated populations may be maintained by natural selection. Life-history models predict that resource levels favour the evolution of increased reproductive output.

There are many genes in this segment of chromosome 3. We believe that *PPARG* is a candidate for dizygotic twinning. PPARγ influences insulin-related effects, lipid metabolism and body mass index, which are all important to the growth process. The two splice variants, only one of which contains the Pro12Ala polymorphism, may explain the fact that we observed a positive association only with the C→T polymorphism. The role of *PPARG* as a 'thrifty' gene seems to be established[14]. Intra-uterine selection may be responsible for the Hardy–Weinberg deviation and we may in fact be dealing with a gene involved in the intrauterine survival of dizygotic twins.

Andreas Busjahn[1,2], Hans Knoblauch[1,2], Hans-Dieter Faulhaber[1], Atakan Aydin[1], Regina Uhlmann[1], Jaakko Tuomilehto[3], Jaakko Kaprio[4], Piotr Jedrusik[5], Andrzej Januszewicz[6], Jan Strelau[7], Herbert Schuster[1,2], Friedrich C. Luft[1] & Bertram Müller-Myhsok[8]

[1]Franz Volhard Clinic, Medical Faculty of the Charité, Humboldt University of Berlin, and Max Delbrück Center, Berlin, Germany. [2]Infogen, a subsidiary of Valigen N.V. [3]National Public Health Institute, Helsinki, Finland. [4]The Finnish Twin Cohort Study, University of Helsinki, Helsinki, Finland. [5]Warsaw Medical University, [6]National Institute of Cardiology, and [7]University of Warsaw, Warsaw, Poland. [8]Bernhard Nocht Institute for Tropical Medicine, Germany. Correspondence should be addressed to F.C.L. (e-mail: luft@fvk-berlin.de).

1. Knoblauch, H. *et al. Arterioscler. Thromb. Vasc. Biol.* **19**, 2940–2944 (1999).
2. Fajas, L. *et al. J. Biol. Chem.* **272**, 18779–18789 (1997).
3. Landy, H.J. & Keith, L. *Hum. Reprod. Update* **4**, 177–183 (1998).
4. Kelly, M.P. *et al. Fertil. Steril.* **56**, 221 (1991).
5. Milad, M.P., Klock, S.C., Moses, S. & Chatterton, R. *Hum. Reprod.* **13**, 2296–2300 (1998).
6. Lewis, C.M., Healey, S.C. & Martin, N.G. *Am. J. Med. Genet.* **61**, 237–246 (1996).
7. Weinberg, W. *Arch. Rass. Ges. Biol.* **6**, 28 (1909).
8. Clark, P. & Martin, N.G. *Hum. Genet.* **61**, 171–174 (1982)
9. Wyshak, G. & White, C. *Am. J. Public Health* **55**, 1586 (1965).
10. Nielsen, D.M. *et al. Am. J. Hum. Genet.* **63**, 1531 (1998).
11. Matte, U. *et al. Acta Genet. Med. Gemellol.* **45**, 431 (1996).
12. Eriksson, A.W. *Hum. Hered.* **23**, 511–534 (1973).
13. Lummaa, V. *et al. Nature* **394**, 533–534 (1998).
14. Kerstein, S., Desvergne, B. & Wahli, W. *Nature* **405**, 421–424 (2000).

Genetic Influences on Baroreflex Function in Normal Twins

Jens Tank, Jens Jordan, Andre Diedrich, Mandy Stoffels, Gabriele Franke, Hans-Dieter Faulhaber, Friedrich C. Luft, Andreas Busjahn

Abstract—Blood pressure and heart rate are strongly influenced by genetic factors; however, despite the pivotal role of genetics in short-term cardiovascular regulation, little is known about the genetic contribution to baroreflex function. We assessed genetic influence on baroreflex sensitivity (BRS) in 149 twin pairs (88 monozygotic of age 33±13 years and BMI 23±4 kg/m^2 and 61 dizygotic of age 33±11 years and BMI 24±4 kg/m^2). ECG and finger arterial blood pressures were measured continuously under resting conditions. BRS values were calculated by use of cross-spectral analysis (baroreflex slope calculated as mean value of transfer function between systolic blood pressure and the R-R interval in the low-frequency band [BRSLF] and baroreflex slope calculated as the mean value of transfer function between systolic blood pressure and R-R interval in the respiratory frequency band [BRSHF]) and the sequence technique (BRS+, BRS-). Heritability (h^2) was estimated with a path-modeling approach. BRS values did not differ significantly between groups (monozygotic, BRSLF, 17±13; BRSHF, 21±18; BRS+, 19±16; and BRS-, 21±15, and dizygotic, BRSLF, 16±9; BRSHF, 20±14; BRS+, 18±10; and BRS-, 20±11 ms/mm Hg), and were significantly correlated ($P<0.001$). When variances and covariances for monozygotic and dizygotic twins were compared, significant correlations were found for BRS in monozygotic (range, $r=0.38$ to 0.48) but not in dizygotic twin pairs ($r=-0.03$ to 0.09). Thus, BRS is heritable; the variability can be explained by genetic influences ($P<0.01$; h^2 range, 0.36 to 0.44). The genetic influence on BRS remained strong after correction for BMI and blood pressure. Therefore, BRS is strongly genetically determined, probably by different genes than are resting blood pressure and BMI. **(*Hypertension*. 2001;37:907-910.)**

Key Words: blood pressure ■ function, autonomic ■ analysis, spectral ■ baroreflex ■ twins ■ genetics

The baroreflexes play a pivotal role in short-term blood pressure (BP) and heart rate regulation.[1] Carotid and aortic baroreceptors sense changes in stretch that result from BP alterations.[2,3] The signal generated in these receptors travels to cardiovascular control centers in the brain stem. This afferent input results in counterregulatory adjustments of sympathetic and parasympathetic tone and prevents excessive fluctuations in BP. Interruption of the afferent arc (baroreflex failure) or the efferent arc (autonomic failure) is associated with extremely labile BP and heart rate.[4,5] Considering the profound effect of complete baroreflex dysfunction on BP and heart rate, even mild abnormalities in baroreflex function could result in substantial changes in cardiovascular regulation. Several studies suggested that baroreflex abnormalities are an important contributing factor to the pathogenesis of arterial hypertension.[1,6–10] Baroreflex disturbances are associated with increased cardiovascular morbidity and mortality.[11] Thus, defining the mechanisms that affect baroreflex function could have an important effect on our understanding of cardiovascular diseases.[7] Recent studies suggested that baroreflex function is influenced by genetic factors.[7,12,13] However, the magnitude of the genetic contribution to baroreflex function is not known. We estimated the magnitude of the genetic effect on baroreflex function in a cohort of normal twins. We tested whether baroreflex sensitivity is influenced by different genes than resting BP and body mass index (BMI), which are known to influence baroreflex function.

Methods

Subjects

We investigated the heritability of baroreflex sensitivity in 149 twin pairs (88 monozygotic and 61 dizygotic). Twin pairs were recruited by advertisement in public print media. All of these normal German twin pairs underwent a medical history taking and physical examination before the study. Persons on ingested medications were excluded from study. Hours of vigorous physical activity per week were estimated by questionnaire. Zygosity was determined by use of 5 microsatellite markers coamplified by polymerase chain reaction. Written informed consent was obtained before study entry as required by the institutional review board.

Study Protocol

Studies were conducted in a quiet room at 20°C during morning hours with the subject in a semi-supine body position. Five-minute recordings were obtained after 10 minutes of rest. BP was measured in the nondominant arm by automated oscillometric device (Dinamap) as well as continuously by Finapres (Ohmeda) BP monitor attached to the middle finger of the right hand. The subject's right

Received June 7, 2000; first decision July 3, 2000; revision accepted September 8, 2000.

From the Clinical Research Center, Franz Volhard Clinic and Max Delbrück Center for Molecular Medicine, Medical Faculty of the Charité, Humboldt University, Berlin, Germany, and the Autonomic Dysfunction Service, Vanderbilt University, Nashville, Tenn (A.D.).

Correspondence to Friedrich C. Luft, MD, Clinical Research Center, Franz-Volhard-Clinic, Humboldt University, Wiltbergstrasse 50, 13125 Berlin, Germany. E-mail luft@fvk-berlin.de

Hypertension **is available at http://www.hypertensionaha.org**

hand was kept at heart level. ECG was recorded continuously with a modified standard lead to optimize the R peak. Respiratory activity was obtained with a pneumobelt. Data were analog-digital converted (ECG 1 kHz, BP 100 Hz). Peak detection (R peak, systolic BP [SBP] and diastolic BP) was realized offline with PV-wave software (VisualNumerics).

Baroreflex-Sequence Technique

Spontaneous baroreflex slope (BRS) was calculated as slope of the linear regression lines between SBP and the subsequent R-R intervals (within the same or the next heart beat) values by use of the sequence technique. Sequences with ≥3 intervals, 0.5-mm Hg BP changes, and 5-ms R-R interval changes were analyzed only if correlation coefficients were >0.85. BRS was calculated as mean value of significant slopes obtained.

Baroreflex–Cross-Spectral Analysis

Power spectral analysis has provided useful information about the temporal fluctuations of different hemodynamic parameters, such as heart rate variability.[14] Cross spectra are used to capture interrelationships between parameters in the time and frequency domain. Therefore, we calculated the power spectra of SBP and R-R interval time series with fast Fourier transformation (segment length, 256 s; resampling with 4 Hz; resolution, 0.004 Hz) and the cross spectra.[15] Baroreflex gain was determined to be the mean value of the transfer function in the low- and high-frequency bands. BRS was considered significant if the coherence in the analyzed frequency band was >0.8.

Statistics and Quantitative Genetics

Statistical analysis was conducted by use of the SPSS program. All data are expressed as mean±SD. Relationship between parameters was assessed by linear regression analysis. Interindividual differences of mean group values were tested with unpaired *t* test. A value for $P<0.05$ was considered to be statistically significant. Parameters of the quantitative genetic models were estimated by structural equation modeling by use of the MX program developed by Neale.[16] Variability of any given phenotype within a population can be decomposed into genetic influences (VaraddGen), environmental influences shared by the twins within a family (VarsharedEnv) and effects of random environment (Varenv), as follows:

Var=VaraddGen+VarsharedEnv+Varenv.

For MZ and DZ, the covariance of their phenotype is given by:

CovMZ=VaraddGen+VarsharedEnv;

CovDZ=0.5 VaraddGen+VarsharedEnv.

Heritability analysis in twin studies can estimate additive components of genetic variability as well as two environmental influences, shared and nonshared environmental influences.[17] These values estimate the relative amount of the influence of the variable on interindividual differences up to a sum of 1. Genetic and environmental effects were estimated by the best-fit model as selected by χ^2 value. Adjustments of baroreflex slopes for age, BMI, and BP were done by multiple linear regression with unstandardized residuals. In case of significant deviations from normal distribution, appropriate transformations were applied.

TABLE 1. Demographic Data of the Twin Pairs

	Twins	
Parameter	Monozygotic	Dizygotic
No.	176	122
Age, y	33±13	33±11
Gender (F/M), n	116/60	82/40
Height, cm	169±9	170±9
Weight, kg	67±11	70±15
BMI, kg/m^2	23.0±3.5	23.8±4.0
SBP, mm Hg	125±16	123±13
DBP, mm Hg	72±11	73±10
Heart rate, bpm	71±11	69±12

Values are mean±SD.

TABLE 2. Group Mean Values of Baroreflex Slopes for Monozygotic and Dizygotic Twins Estimated With Cross-Spectral Analysis and Sequence Technique

	Twins	
Parameter	Monozygotic	Dizygotic
BRSLF	17±13	16±9
BRSHF	21±18	20±14
BRS+	19±16	18±10
BRS−	21±15	20±11

Values are mean±SD.

Results

Monozygotic and dizygotic twins had similar age, BMI, resting BP, and resting heart rate (Table 1). Baroreflex slopes (Table 2) determined with different methods were highly correlated (Figure 1). Large interindividual variability existed in baroreflex sensitivity. For example, baroreflex slopes determined by the sequence technique ranged from 2 to 82 ms/mm Hg. Baroreflex slopes determined with sequence and cross-spectral techniques were similar in monozygotic and dizygotic twins. A significant decrease was seen in baroreflex sensitivity with age (Figure 2). Distribution of baroreflex sensitivities resembled the pattern described earlier in normal subjects of similar age.[18] Baroreflex sensitivity was negatively correlated with BMI and resting BP. Daily physical activity was positively correlated with baroreflex sensitivity (Table 3). No gender differences occurred in baroreflex sensitivity.

Baroreflex sensitivity was significantly correlated in monozygotic twin pairs but not in dizygotic twin pairs (Table 4). Because age had a strong effect on baroreflex sensitivity, baroreflex sensitivities were adjusted for age for heritability analysis. Heritability of baroreflex sensitivity adjusted for age ranged from 0.36 to 0.44. These results suggest a strong genetic influence on baroreflex sensitivity. Heritability of baroreflex sensitivity deter-

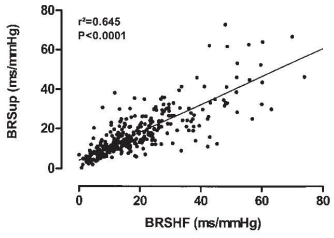


Figure 1. Linear regression analysis between baroreflex sensitivity determined by different methods. BRSup indicates baroreflex slope calculated by use of the sequence technique for increasing SBP; BRSHF, baroreflex slope calculated as mean value of the transfer function between SBP and the R-R interval in the respiratory-frequency band (0.15 to 0.4 Hz).

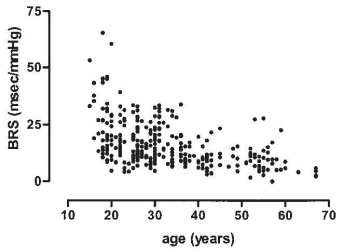


Figure 2. Baroreflex sensitivity as a function of age. BRSLF indicates baroreflex slope calculated as mean value of the transfer function between SBP and the R-R interval in the low-frequency band (0.04 to 0.15 Hz).

mined by cross-spectral analysis and sequence technique (upslopes) was attenuated only slightly after adjustment for BMI and resting BP. Heritability of BRS determined by sequence technique with downslopes was markedly reduced after adjustment for BMI and resting BP.

Discussion

The main finding of the present study is that BRS in the normal human is strongly influenced by genetic variance. The heritability estimate was only slightly attenuated after correction for BMI and BP. Therefore, BRS seems, in part, to be influenced by different genes than are resting BP and BMI. This finding is of potential clinical relevance because abnormalities in BRS have been implicated in the pathogenesis of arterial hypertension and are associated with increased cardiovascular mortality.[1,6–11]

Few human studies have addressed the issue of whether baroreflex function is influenced by genetic factors. None of these studies provided estimates of the magnitude of the genetic effect on BRS. For example, normotensive and borderline hypertensive subjects with a family history of hypertension exhibited a decrease in BRS compared with normotensives without a family history of hypertension.[19] In one population-based study from Finland, a common genetic polymorphism in the promoter and in the coding region of the aldosterone-synthase gene was found to influence baroreflex sensitivity.[20] This effect appeared to be stronger in younger than in older subjects. In contrast, no association was found for BRS with insertion/deletion polymorphism of the angiotensin-converting enzyme gene or M235T variants of the angiotensinogen gene. The hypothesis that the renin-angiotensin-aldosterone system contributes to BRS is supported by the observation that BRS can be improved with angiotensin-converting enzyme inhibition.[18] The effect of numerous genes on BRS was studied in animals.[21–24] These genes are possible candidate genes for future studies in humans.

TABLE 3. Cross Correlation Between BRSLF, BMI, BP, and Physical Activity

Parameters	r^2 (Pearson)	P
BRSLF vs BMI	−0.193	<0.001
BRSLF vs SBP	−0.335	<0.001
BRSLF vs DBP	−0.319	<0.001
BRSLF vs Physical Activity	0.166	<0.02

TABLE 4. Intraclass Correlation Coefficients (r) and Heritability (h^2) for Monozygotic and Dizygotic Twins Before and After Correction for BMI and Resting BP (c) for Calculated Baroreflex Slopes

Parameter	r MZ	r DZ	h^2	r MZc	r DZc	h^2 c
BRSLF	0.48	0.05	0.43*	0.43	0.13	0.41*
BRSHF	0.44	−0.03	0.40*	0.37	0.08	0.35*
BRS+	0.46	0.03	0.42*	0.40	0.16	0.39*
BRS−	0.38	0.09	0.36*	0.29	0.20	0.18

MZ indicates monozygotic; DZ, dizygotic twins.
*P<0.01.

We assessed only the genetic contribution to baroreflex control of heart rate. Baroreflex control of heart rate is mainly achieved through changes in parasympathetic tone. In contrast, baroreflex control of vascular tone is a function of the sympathetic nervous system.[1,13,15] Changes in baroreflex control of heart rate are not always associated with similar changes in regulation of vascular tone.[13,25] Thus, the results of the present study cannot be interpreted to indicate a genetic effect on vascular tone exercised by the sympathetic nervous system. The genetic effect on parasympathetic and on sympathetic regulation by the baroreflex could be independent, in part.[25–27] Yet, in an earlier study, family history of hypertension was associated with attenuated baroreflex-mediated reduction in sympathetic nerve traffic.[13] An interesting case report described a symptomatic failure of the baroreceptor BP buffering mechanism in a woman with familial aniridia. Her baroreceptor cardiac inhibition was intact.[25] Furthermore, the parasympathetic component of the arterial baroreflex becomes impaired with advancing age. Baroreflex control of sympathetic outflow to the peripheral circulation, as assessed by direct measurements of muscle sympathetic nerve activity, can be well maintained in healthy individuals even into the seventh decade of life.[27]

Characterization of the baroreflex control of sympathetic outflow in large-scale genetic studies will be difficult because direct measurement of muscle sympathetic nerve activity is complicated, involves intravenous infusions of vasoactive medications, and is established at only a few centers. From a clinical standpoint, characterization of the genes that influence baroreflex control of heart rate may be more urgently needed because of the wealth of data implicating BRS as a prognostic marker.[11]

Twin studies have been extensively used to characterize the interaction of genetic and environmental factors on cardiovascular phenotypes.[28] The twin approach allows detection and quantification of genetic effects in relatively small subject groups.[17] One potential limitation of the present study is that we characterized genetics of baroreflex function in a cohort of healthy subjects. However, genes involved in monogenic diseases were shown to act as quantitative trait loci in the general population, which supports the close relationship between physiological and pathological processes.[29,30] Thus, the genetic effect on BRS also may be important in the pathogenesis of cardiovascular disorders. These genetic

factors may modulate the effect of aging, BMI, physical activity, and BP on baroreflex function. Baroreflex slope calculated as the mean value of the transfer function between SBP and the R-R interval in the low-frequency band, baroreflex slope calculated as the mean value of the transfer function between SBP and the R-R interval in the respiratory-frequency band, and BRS+ showed strong evidence for heritability. When corrections for resting BP and BMI were made, the same degree of heritability was still evident. Only the heritability estimated for BRS- was attenuated after adjustment for BMI and resting BP. These findings support the interpretation that BRS is controlled by distinct genetic factors independent of those influencing BMI and resting BP.

We conclude that BRS is strongly influenced by genetic factors. BRS seems to be, at least in part, influenced by different genes than BMI and resting BP. BRS may thus be an important additional intermediate phenotype in genetic studies on cardiovascular regulation. Furthermore, elucidation of the genes influencing BRS may provide new insight into cardiovascular regulation and pathogenesis of cardiovascular diseases.

References

1. Eckberg DL, Sleight P. Hypertension. In: *Monographs of the Physiological Society (43): Human Baroreflex in Health and Disease.* Oxford, UK: Clarendon Press, 1992;327 366.
2. Drummond HA, Price MP, Welsh MJ, Abboud FM. A molecular component of the arterial baroreceptor mechanotransducer. *Neuron.* 1998;21: 1435 1441.
3. Eckberg DL, Sleight P. Baroreflex anatomy. In: *Monographs of the Physiological Society (43): Human Baroreflex in Health and Disease.* Oxford, UK: Clarendon Press, 1992:19 57.
4. Jordan J, Shannon JR, Black B, Costa F, Ertl AC, Furlan R, Biaggioni I, Robertson D. Malignant vagotonia due to selective baroreflex failure. *Hypertension.* 1997;30:1072 1077.
5. Robertson D, Hollister AS, Biaggioni I, Netterville JL, Mosqueda-Garcia R, Robertson RM. The diagnosis and treatment of baroreflex failure. *N Engl J Med.* 1993;329:1449 1455.
6. Imai Y, Aihara A, Ohkubo T, Nagai K, Tsuji I, Minami N, Satoh H, Hisamichi S. Factors that affect blood pressure variability: a community-based study in Ohasama, Japan. *Am J Hypertens.* 1997;10: 1281 1289.
7. Parmer RJ, Cervenka JH, Stone RA. Baroreflex sensitivity and heredity in essential hypertension. *Circulation.* 1992;85:497 503.
8. Ditto B, France C. Carotid baroreflex sensitivity at rest and during psychological stress in offspring of hypertensives and non-twin sibling pairs. *Psychosom Med.* 1990;52:610 620.
9. Ookuwa H, Takata S, Ogawa J, Iwase N, Ikeda T, Hattori N. Abnormal cardiopulmonary baroreflexes in normotensive young subjects with a family history of essential hypertension. *J Clin Hypertens.* 1987;3: 596 604.
10. Piccirillo G, Viola E, Nocco M, Durante M, Tarantini S, Marigliano V. Autonomic modulation of heart rate and blood pressure in normotensive offspring of hypertensive subjects. *J Lab Clin Med.* 2000;135:145 152.
11. La Rovere MT, Bigger JT, Marcus FI, Mortara A, Schwartz PJ. Baroreflex sensitivity and heart-rate variability in prediction of total cardiac mortality after myocardial infarction: ATRAMI (Autonomic Tone and Reflexes After Myocardial Infarction) Investigators. *Lancet.* 1998; 351:478 484.
12. Weinstock M, Gorodetsky E. Comparison of the effects of angiotensin II, losartan, and enalapril on baroreflex control of heart rate in conscious rabbits. *J Cardiovasc Pharmacol.* 1995;25:501 507.
13. Yamada Y, Miyajima E, Tochikubo O, Matsukawa T, Shionoiri H, Ishii M, Kaneko Y. Impaired baroreflex changes in muscle sympathetic nerve activity in adolescents who have a family history of essential hypertension. *J Hypertens Suppl.* 1988;6:S525 S528.
14. Task Force of the European Society of Cardiology and the North American Society of Pacing and Electrophysiology. Heart rate variability: standards of measurement, physiological interpretation, and clinical use. *Eur Heart J.* 1996;17:354 381.
15. DeBoer RW, Karemaker JM, Strackee J. Hemodynamic fluctuations and baroreflex sensitivity in humans: a beat-to-beat model. *Am J Physiol.* 1987;253:680 689.
16. Neale MC: Mx: Statistical modeling. Box 126 MCV, Richmond, VA 23298: Department of Psychiatry. 4th edition 1997.
17. Neale MC, Cardon LR. Methodology for genetic studies of twins and families. Dordrecht, Netherland: Kluwer Academic Publishers; 1992:496.
18. Tank J, Baevski RM, Fender A, Baevski AR, Graves KF, Ploewka K, Weck M. Reference values of indices of spontaneous baroreceptor reflex sensitivity. *Am J Hypertens.* 2000;13:268 275.
19. Iwase N, Takata S, Okuwa H, Ogawa J, Ikeda T, Hattori N. Abnormal baroreflex control of heart rate in normotensive young subjects with a family history of essential hypertension. *J Hypertens Suppl.* 1984;2: S409 S411.
20. Ylitalo A, Airaksinen KE, Hautanen A, Kupari M, Carson M, Virolainen J, Savolainen M, Kauma H, Kesaniemi YA, White PC, Huikuri HV. Baroreflex sensitivity and variants of the renin angiotensin system genes. *J Am Coll Cardiol.* 2000;35:194 200.
21. Mansier P, Medigue C, Charlotte N, Vermeiren C, Coraboeuf E, Deroubai E, Ratner E, Chevalier B, Clairambault J, Carre F, Dahkli T, Bertin B, Briand P, Strosberg D, Swynghedauw B. Decreased heart rate variability in transgenic mice overexpressing atrial beta 1-adrenoceptors. *Am J Physiol.* 1996;271:H1465 H1472.
22. Miyajima E, Bunag RD. Impaired sympathetic baroreflexes in prehypertensive Dahl hypertension-sensitive rats. *Clin Exp Hypertens A.* 1986;8: 1049 1061.
23. Madeddu P, Salis MB, Emanueli C. Altered baroreflex control of heart rate in bradykinin B2-receptor knockout mice. *Immunopharmacology.* 1999;45:21 27.
24. Hilbert P, Lindpaintner K, Beckmann JS, Serikawa T, Soubrier F, Dubay C, Cartwright P, De Gouyon B, Julier C, Takahasi S, et al. Chromosomal mapping of two genetic loci associated with blood-pressure regulation in hereditary hypertensive rats [see comments]. *Nature.* 1991;353:521 529.
25. Julu PO, McCarron MO, Hansen S, Barnes A, Jamal GA, Ballantyne JP. Selective defect of baroreflex blood pressure buffering with intact cardioinhibition in a woman with familial aniridia. *Neurology.* 1997;49: 1705 1708.
26. Furlan R, Jacob G, Snell M, Robertson D, Porta A, Harris P, Mosqueda GR. Chronic orthostatic intolerance: a disorder with discordant cardiac and vascular sympathetic control. *Circulation.* 1998;98:2154 2159.
27. Ebert TJ, Morgan BJ, Barney JA, Denahan T, Smith JJ. Effects of aging on baroreflex regulation of sympathetic activity in humans. *Am J Physiol.* 1992;263:H798 H803.
28. Busjahn A, Voss A, Knoblauch H, Knoblauch M, Jeschke E, Wessel N, Bohlender J, McCarron J, Faulhaber HD, Schuster H, Dietz R, Luft FC. Angiotensin-converting enzyme and angiotensinogen gene polymorphisms and heart rate variability in twins. *Am J Cardiol.* 1998;81: 755 760.
29. Knoblauch H, Muller MB, Busjahn A, Ben-Avi L, Bahring S, Baron H, Heath SC, Uhlmann R, Faulhaber HD, Shpitzen S, Aydin A, Reshef A, Rosenthal M, Eliav O, Muhl A, Lowe A, Schurr D, Harats D, Jeschke E, Friedlander Y, Schuster H, Luft FC, Leitersdorf E. A cholesterol-lowering gene maps to chromosome 13q. *Am J Hum Genet.* 2000;66: 157 166.
30. Busjahn A, Knoblauch H, Faulhaber HD, Boeckel T, Rosenthal M, Uhlmann R, Hoehe M, Schuster H, Luft FC. QT interval is linked to 2 long-QT syndrome loci in normal subjects. *Circulation.* 1999;99: 3161 3164.